EFFECT OF CO-ADMINISTRATION OF AQUEOUS EXTRACT OF *Hibiscus Sabdariffa Linn (Malvaceae)* CALYX AND VITAMIN E ON CARBAMAZEPINE-INDUCED TESTICULAR CHANGES IN ADULT WISTAR RATS

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DECLARATION

I declare that the work in this thesis, entitled 'evaluation of co-administration of *Hibiscus sabdariffa linn* and vitamin E on carbamazepine-induced testicular changes in adult male Wistar rats' has been performed by me in the Department of Human Physiology, Ahmadu Bello University, Zaria under the supervision of Dr Muhammed U. Kawu and Dr Y. Tanko.

The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

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CERTIFICATION

This thesis, entitled "EVALUATION OF CO-ADMINISTRATION OF *Hibiscus Sabdariffa Linn* AND VITAMIN E ON CARBAMAZEPINE-INDUCED TESTICULAR CHANGES IN ADULT MALE WISTAR RATS" by Ibrahim SULEIMAN meets the regulations governing the award of the degree of Master of Science in Human Physiology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to Almighty Allah (S.W.T) for his assistance and protection through the course of this project work, without him I am nobody and also to my parents, Mal. Salami Ibrahim and Mallama Ibrahim Hajara, for their invaluable support throughout my endeavours and to the entire physiologists in the world.

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ABSTRACT

The antioxidant potential of many flavonoid containing plants is increasingly being exploited in therapy. *Hibiscus sabdariffa* (HS) is one of the most widely used nutraceuticals used traditionally to combat various illnesses due to its high flavonoid contents. The present study was aimed at evaluating the ameliorative effect of co-administration of HS and vitamin E on sub-chronic carbamazepine (CBZ)-induced alterations in semen characteristics. Thirty young male Wistar rats were divided at random into five groups containing 6 rats per groups. Group I and II were administered normal saline and carbamazepine (20 mg /kg), respectively, while group III was treated with CBZ and aqueous extract of *Hibiscus Sabdariffa* (200 mg/kg); group IV was treated with CBZ and vitamin E (VE) 30 minutes after treatment with CBZ. The regimens were administered orally by gavage once daily for a period of 8 weeks. At the end of the treatment, four animals from each group were randomly selected and sacrificed by jugular fenestration. The result obtained showed that CBZ caused a pronounced reduction in sperm concentration, motility, testicular histological damage, sperm morphological abnormalities, and increased the testicular MDA concentration. In control group, sperm count was 61.50 ± 1.44 $x10^6$/ml, and 49.75 ± 3.19 $x10^6$/ml, 71.25 ± 7.08 $x10^6$/ml, 63.00 ± 2.67 $x10^6$/ml and 89.00 ± 9.85 $x10^6$/ml for CBZ, CBZ + HS, CBZ + VE and CBZ + HS + VE groups respectively. Sperm progressive motility was 61.25 ± 3.14%, 42.50 ± 4.78%, 66.25 ± 6.88%, 67.50 ± 6.29% and 82.50 ± 4.78% for the control, CBZ, CBZ + HS, CBZ + VE and CBZ + HS + VE groups respectively, sperm non-progressive motility was 26.25 ± 2.39%, 30.00 ± 4.56%, 20.00 ± 3.53%, 17.50 ± 2.50%, and 7.50 ± 1.44% for the control, CBZ, CBZ + HS, CBZ + VE and CBZ + HS + VE groups respectively, while, non-motile sperm cells was 22.50 ± 4.78%, 27.50 ± 1.44%, 13.75 ± 3.75%, 15.00 ± 5.00%, and 10.00 ± 3.53% for the control, CBZ,

CBZ + HS, CBZ + VE and CBZ + HS + VE groups respectively. Serum testosterone concentration in control group was 6.15 ± 0.22 µg/ml, and 5.05 ± 0.06 µg/ml, 4.25 ± 0.34 µg/ml, 4.70 ± 0.16 µg/ml, and 5.97 ± 0.31 µg/ml for CBZ, CBZ + HS, CBZ + VE and CBZ + HS + VE groups respectively. The testicular MDA concentration was 0.05 ± 0.01 nmoL/mg, 0.17 ± 0.012 nmoL/mg, 0.04 ± 0.02 nmoL/mg, 0.06 ± 0.00 nmoL/mg and 0.02 ± 0.00 nmoL/mg for the control, CBZ, CBZ + HS, CBZ + VE and CBZ + HS + VE groups respectively. However, HS, VE and their combination, ameliorated the alterations in the semen characteristics and reduced the testicular MDA concentration induced by CBZ. The study concludes that HS, VE and their combination mitigate the alterations in semen characteristics and testicular lipoperoxidation caused by CBZ in adult male Wistar rats.

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LIST OF ABBREVIATIONS/SYMBOLS

(O_2^-) - Superoxide anions radicals

AA- Ascorbic acid

ADHD - Attention-deficit hyperactivity disorder

ADP - Adenosine diphosphate

AEDs - Antiepileptic drugs

ALP - Alkaline phosphatase

ANOVA - Analysis of variance

$AUC_{O\text{-}alpha}$ - Area under the plasma concentration-time curve

BHA - Butylated hydroxyl anisole

BHT - Butylatedhydroxy toluene

cAMP - Cyclic adenosine monophosphate

CARET - Carotene and Retinol Efficacy Trial

CAT - Catalase

CBZ - Carbamazepine

CD36 - Cluster of differentiation 36

C_{max} - Maximum or peak concentration

CO_2 - Carbon mono-oxide

COX - Cyclo-oxygenase

CRH - Corticotropin-releasing hormone

CTGF - Connective tissue growth factor

CYP3A4 - Cytochrome P450 3A4

CYP450 - Cytochrome P450

DHEAS - Dehydroepiandrosterone sulfate

DNA - Deoxyribonucleic acid

EAE - Experimental allergic encephalomyelitis

EDTA - Ethylene di-amine tetra-acetic acid

Fe^{2+} - Ferrous (Iron II ion)

Fe^{3+} - Ferric (Iron III ion)

FSH - Follicle-stimulating hormone

GABA - Gamma amino butyric acid

GHRH - Growth hormone releasing hormone

GnRH - Gonadotropin-releasing hormone

GOT - Glutamic oxaloacetic transaminase

GPT - Glutamic-pyruvate transaminase

GPx - Glutathione peroxidase

HS - *Hibiscus sabdariffa*

H_2O_2 - Hydrogen peroxide

HCl - Hydrochloric acid

HL-60 - Cells Human leukemia cells

HMG-CoA - 3-hydroxy-3-methyl-glutaryl CoA reductase

IOM - Institute of Medicine

IU - International unit

IVT - Illini Variable Temperature

L - Linn

LDL - Low density lipoprotein

MDA - Malondialdehyde

MS - Multiple sclerosis

NaCl - Sodium chloride

NBT - Nitro-blue tetrazolium

OH^- - Hydroxyl ion

ORAC - Oxygen radical absorbance capacity

PBS - Phosphate buffer solution

PCEs - Polychromatic erythrocytes

PHE - Phenytoin

PKC - Protein kinase C

POIS - Post orgasmic illness syndrome

PSS - Physiological saline solution

PUFAs - Polyunsaturated fatty acids

RAW264.7 - cells Mouse macrophage cell line

RBC - Red blood count

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

SHBG - Sex hormone-binding globulin

SOD - Superoxide dismutase

SR-A - Scavenger receptor class A

SREBPs - Sterol regulatory element-binding proteins

$t_{1/2}a$ - Half- life alpha

TBA - Thiobabuturic acid

t-BHP - Tert-Butyl hydroperoxide

TBHQ - Tertiary butylatedhydroxyquinone

TCA - Trichloroacetate

T_{max} - Time to maximum concentration

TRH - Thyrotropin-releasing hormone

TSH - Thyrotropin

U.S - United state

UA - Uric acid

USDA - United States Department of Agriculture

α - Alpha

γ - Gamma

CHAPTER ONE

1.0 INTRODUCTION

Carbamazepine is one of the most commonly used antiepileptic drugs. In men with epilepsy, long-term use of liver enzyme inducing antiepileptic drugs (AEDs), is associated with increased serum concentration of sex hormone-binding globulin and reduced bioactive serum testosterone, which may affect reproductive function (Isojarvi *et al.,* 2005). In addition, the use of certain antiepileptic drugs may reduce sperm motility, induce sperm abnormalities and decrease testicular volume. Change in sperm quality can obviously have a direct effect on fertility in men (Roste *et al.,* 2003; Isojarvi *et al.,* 2004).

Hibiscus sabdariffa L. is used in folk medicine against many complaints that include high blood pressure, liver diseases and fever (Dalziel, 1973; Wang *et al.,* 2000; Odigie *et al.,* 2003 b; Ali *et al.,* 2005). In Nigeria, a decoction of the seeds is given to augment or induce lactation in poor milk let-down (Okasha *et al.,* 2008). *Hibiscus sabdariffa L.* is taken as a common local drink, popularly known as "*zobo*" in Nigeria. It is cultivated for leaf, fleshy calyx, seed or fibre according to the respective properties of the two major varieties variety. ruber (red) and variety intermedius (green) (Dalziel, 1973). The thick red and fleshy cup-shapes calyces of the flower are consumed worldwide as a cold beverage and as a hot drink (sour tea) (Morton, 1987). It is known as roselle or red sorrel (English), *karkade* (Arabic), *yakuwa* (Hausa), *amukan* (Yoruba) and *okworo ozo* (Ibo). *Hisbiscus sabdariffa Linn* is a herb belonging to the Malvaceae family and it is grown in Central and West Africa, South-East Asia, and elsewhere in parts of West Indies, Jamaica and Central America. In Africa, roselle is frequently cooked as a side dish, eaten with pulverized peanuts. For stewing as

sauce or filling for tarts or pies, the products were indistinguishable from cranberry sauce in taste and appearance (Monton, 1987; Olaleye, 2007).

Hibiscus sabdariffa L. is an important source of vitamins, minerals, and bioactive compounds, such as organic acids, phytosterols, and polyphenols, some of them with antioxidant properties. The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-glucoside, sambubioside, and cyanidin-3- sambubioside; other flavonoids like gossypetin, hibiscetin, and their respective glycosides; protocatechuic acid, eugenol, and sterols like -sitoesterol and ergoesterol (Ali-Bradeldin, 2005). *Hibiscus sabdariffa L.* is used as a source of food and beverages in local communities in Africa and other parts of the world. *Hibiscus sabdariffa L.*is reported to have antioxidant effect on some haematological indices and in alleviating the toxicity induced by chronic administration of sodium nitrate in Wistar rats (Bako *et al.,* 2009).

In India, Africa and Mexico, roselle plants are valued in native medicine. Infusions of the leaves or calyces are regarded as diuretic, cholerectic, febrifugal and hypotensive, decreasing the viscosity of the blood and stimulating intestinal peristalsis. Pharmacognosists in Senegal recommend roselle extract for lowering blood pressure. Sharaf (1962), confirmed the hypotensive activity of the calyces and found them antispasmodic, anthelmintic and antibacterial as well. In 1964, the aqueous extract was found effective against *Ascaris gallinarum* in poultry (Monton, 1987). Sharaf *et al*., (1962) showed that both the aqueous extract and the colouring matter of the calyces are lethal to *Mycobacterium tuberculosis* (Monton, 1987). In experiments with domestic fowl, roselle extract decreased the rate of absorption of alcohol and so lessened its effect on the system (Monton, 1987). In Guatemala, roselle "ade" is a favorite remedy for the after-effects of drunkenness. The

heated leaves are applied to cracks in the feet and on boils and ulcers to speed maturation of new cells. A lotion made from the leaves is used on sores and wounds (Builders *et al*., 2013). The brownish-yellow seed oil is claimed to heal sores on camels. In India, a decoction of the seeds is given to relieve dysuria, strangury and mild cases of dyspepsia and debility (Monton, 1987).

Vitamin E can protect critical cellular structures against damage both from free radicals such as peroxyl radical, hydroxyl radical, and superoxide, and from oxidation products such as malondialdehyde (MDA) and hydroxynonenal (Erin, *et al.,* 1984). Vitamin E, as an important antioxidant, playing a role in inhibition of mutagen formation and repair of membranes and DNA. Therefore, it has been suggested that vitamin E may be useful in cancer prevention (London, 1985). Vitamin E supplementation in cancer patients showed that vitamin E has an important neuroprotective effect (Pace, 2003). Vitamin E has the ability to protect neuronal tissue in several neurodegenerative disorders, including Alzheimer's disease (Sano *et al.,* 1997).

Oxidative stress is thought to contribute to the development of a wide range of diseases, including Alzheimer's disease (Christen, 2000; Nunomura *et al*., 2006), Parkinson's disease (Wood-Kaczmar *et al*., 2006), diabetes (Giugliano *et al*., 1996; Davì *et al* ., 2005), rheumatoid arthritis (Hitchon and El-Gabalawy, 2004) and neurodegeneration in motor neurone diseases (Cookson and Shaw, 1999). In many of these cases, it is unclear if oxidants trigger the disease, or if they are produced as a secondary consequence of the disease and from general tissue damage. One case in which this link is particularly well-understood is the role of oxidative stress in cardiovascular disease. Here, low-density lipoprotein (LDL)

oxidation appears to trigger the process of atherogenesis, which results in atherosclerosis, and finally cardiovascular disease (Van Gaal *et al*., 2006).

Spermatozoa were the first cell type in which the cellular generation of reactive oxygen was demonstrated. This activity has now been confirmed in spermatozoa from all mammalian species examined including the rat, mouse, rabbit, horse, bull and human being. Under physiological circumstances, cellular redox activity is thought to drive the cAMP-mediated, tyrosine phosphorylation events, associated with sperm capacitation. In addition to this biological role, human spermatozoa also appear to suffer from oxidative stress, with impacts on the normality of their function and the integrity of their nuclear and mitochondrial DNA (Aitken and Baker, 2004). Oxidative damage in DNA can cause cancer. Several antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase. It protects DNA from oxidative stress. It has been proposed that polymorphisms in these enzymes are associated with DNA damage and subsequently, the individual's risk of cancer susceptibility (Khan *et al*., 2010).

A low-calorie diet extends median and maximum lifespan in many animals. This effect may involve a reduction in oxidative stress (López-Lluch *et al*., 2006). While there is some evidence to support the role of oxidative stress in ageing in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Helfand and Rogina, 2003; López-Lluch *et al*., 2006), the evidence in mammals is less clear (Sohal *et al*., 2002). Indeed, a review of experiments in mice concluded that almost all manipulations of antioxidant systems had no effect on aging (Pérez *et al*., 2009). Diets high in fruit and vegetables, which are high in antioxidants, promote health and reduce the effects of aging; antioxidant vitamin supplementation has no detectable effect on the ageing process, so the effects of fruit and

vegetables may be unrelated to their antioxidant contents (Thomas, 2004). One reason for this might be the fact that consuming antioxidant molecules such as polyphenols and vitamin E produces changes in other aspects of metabolism, and perhaps this may be the reason why these compounds are important in human nutrition (Aggarwal and Shishodia, 2006).

1.1 STATEMENT OF RESEARCH PROBLEM

Epilepsy is a major public health problem affecting nearly 50 million people world-wide (Scott *et al.,* 2001). World Health Organization (2004) estimates that 10 million people in Africa live with epilepsy, and 8 million (80%) are not receiving adequate treatment due to high cost of treatment and adverse effects of antiepileptic drugs. The point prevalence of epilepsy varies from 5 to 37 per 1000 in Nigeria (Osuntokun *et al.,* 1982; Akinsulore, 2011). Reproductive endocrine disorders and sexual dysfunction are common in men with epilepsy (Herzog and Fowler, 2005). In addition, these disorders have been frequently associated with long-term treatment with AEDs (Herzog *et al.,* 2004; Isojarvi *et al.,* 2005; Roste *et al.,* 2005). Carbamazepine is a first –line antiepileptic, and is also utilized for treatment of psychiatric disorders such as bipolar affective disorder (Albani *et al.,* 1995), and neuropathic pain (Sindrup and Jensen, 1999). The long-term administration of carbamazepine is a common schedule for the treatment of epilepsy in children and adolescents (Mikkonen *et al.,* 2004), and can provoke hormonal alterations and infertility (Oliva and Miraglia, 2009). The reproductive hormone status differs noticeably in humans, according to the phase considered, that is, in childhood, adolescence and adulthood. Thus, endocrine changes due to long-term carbamazepine treatments may cause a negative impact

on pubertal development and fertility both in boys and young men (Mikkonen *et al.,* 2004). Damage of the seminiferous epithelium, testicular interstitial oedema, reduction of testosterone levels and increase in estradiol levels were observed in rats, treated with carbamazepine since weaning. The association of vitamin E deficiency with impaired male reproduction was established more than three decades ago, and traditionally it is called the " anti-sterility Vitamin" (Oliva and Miraglia, 2009).

1.2 JUSTIFICATION

Various clinical and experimental studies involving the effects of carbamazepine and other antiepileptic drugs on male reproduction have been carried out. They have mainly shown hormonal changes and seminal alterations during adult phase, including reduced sperm motility, sperm morphological alterations and decrease in the sperm concentration (Chen *et al.,* 1992; Soliman and El-Meguid, 1999; Isojarvi *et al.,* 2004). Studies on the use of antioxidants of plant origin and (or) its combination with vitamin E to ameliorate reproductive changes caused by long-term carbamazepine treatment is to the best of our knowledge unavailable. Therefore, this study was designed to evaluate the effect of *H. sabdariffa* alone and its combination with vitamin E on reproductive changes, caused by long-term administration of the anti-epileptic drug, carbamazepine. Such information could provide a cheaper and non-toxic alternative adjunct treatment that could be used to ameliorate reproductive changes, associated with the long-term use of carbamazepine in the chronic management of epilepsy in mature males.

1.3 AIM AND OBJECTIVES

The aim of this study was to determine the effect of *Hibiscus sabdariffa L.* and its combination with vitamin E on carbamazepine-induced reproductive changes and the role of oxidative stress in the pathophysiology of these changes, in adult male Wistar rats.

The objectives of the study are:

1. To determine the effect of carbamazepine administration on testicular and sperm characteristics of adult male Wistar rats.
2. To determine the effect of carbamazepine administration on serum testosterone concentration in adult male Wistar rats.
3. To determine the effect of carbamazepine administration on some testicular biomarkers of oxidative stress (superoxide dismutase, catalase, glutathione peroxide and malondialdehyde).
4. To evaluate the effect of *H. sabdarriffa* and its combination with vitamin E on carbamazepine-induced testicular changes and testicular biomarkers of oxidative stress in male Wistar rats.

1.4 NULL HYPOTHESIS

H_o: *Hibiscus sabdariffa L.* or its combination with vitamin E has no effect on carbamazepine-induced reproductive changes in adult male Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *HIBISCUS SABDARIFFA LINN*



Plate I: *Hibiscus sabdariffa* Linn (Monton, 1987)

Hibiscus sabdariffa linn. is a shrub belonging to the family - Malvaceae. It is thought to be native to Asia (India to Malaysia) or tropical Africa. The plant is widely grown in tropics like Caribbean, Central America, India, Africa, Brazil, Australia, Hawaii, Florida and Philippines as a home garden crop of export, especially in the western parts where it occupies second place area wise after pearl millet followed by sesamum (Gautam, 2004; Leung and Foster, 1996).

True roselle is *Hibiscus sabdariffa* L. (family Malvaceae), and there are 2 main types. The more important economically is *H. sabdariffa* var. *altissima* wester (Plate I), an erected, and sparsely-branched annual plant, which is cultivated for its jute-like fiber in India, the East Indies, and Nigeria and to some extent in tropical America (Julia, 1987). In addition to Roselle, in English-speaking regions, it is called as Rozelle, Sorrel, Red sorrel Jamaica sorrel, Indian sorrel, Sour-sour, Queensland jelly plant, Jelly okra, Lemon bush and Florida cranberry. In North Africa and the near east, Roselle is called *karkade* or *carcade,* and it is known by these names in the pharmaceutical and food-flavouring trades in Europe (Julia, 1987). The plant is about 3.5m tall and has a deep penetrating taproot. It has a smooth or nearly smooth, cylindrical, typically dark green to red stems. Leaves are alternate, 7.5-12.5 cm long, green with reddish vein s and long or short petioles. Leaves of young seedlings and upper leaves of older plants are simple; lower leaves are deeply 3 to even 7-lobed and the margins are toothed. Flowers, borne singly in the leaf axils are up to 12.5 cm wide, yellow of buff with a rose or maroon eye and turn pink as they wither at the end of the day. The typically red calyx consists of 5 large sepals with a collar (epicalyx) of 8-12 slim, pointed bracts around the base. They begin to enlarge at the end of the day, 3.2-5.7 cm long and

fully enclose the fruit. The fruit is a velvety capsule, 1.25-2 cm long, which is green, when immature, 5-valved, with each valve containing 3-4 seeds. The capsule turns brown and splits open when mature and dry. Seeds are kidney-shaped, light-brown, 3-5 mm long and covered with minute, stout and stellate hairs (Julia, 1987).

2.1.1 Food Value of *Hibiscus sabdariffa*

Nutritionists have found roselle calyces as sold in Central American markets to be high in calcium, niacin, riboflavin, iron and other constituents showing in Tables 2.1, 2.2, 2.3 and 2.4 (Julia, 1987).

Food Value Per 100 g of Edible Portion

Table 2.1: The constituents and amount of substances present in *H. sabdariffa* calyces (Julia, 1987).

Constituent	Amount present
Moisture	9.2 g
Protein	1.145 g
Fat	2.61 g
Fiber	12.0 g
Ash	6.90 g
Calcium	1,263 mg
Phosphorus	273.2 mg
Iron	8.98 mg
Carotene	0.029 mg
Thiamine	0.277 mg
Niacin	3.765 mg

Ascorbic Acid	6.7 mg

Table 2.2: The constituents and amount of substances present in *H. sabdariffa* leaves (Julia, 1987).

Constituent	**Amount present in percentage**
Moisture	86.2%
Protein	1.7-3.2%
Fat	1.1%
Carbohydrates	10%
Ash	1%
Calcium	0.18%
Phosphorus	0.04%
Iron	0.0054%
Malic Acid	1.25%

Table 2.3: The constituents and amount of substances present in *H. sabdariffa* root (Julia, 1987).

Constituent	**Amount present in percentage**
Moisture	12.9%
Protein	3.29%
Fatty Oil	16.8%
Cellulose	16.8%
Pentosans	15.8%

Starch	11.1%

Table 2.4: Showing the constituents and amount of amino acids (mg/g of dry calyx) substances present in *H. sabdariffa* (Julia, 1987).

Amino acid	**Amount present**
Arginine	3.6
Cystine	1.3
Histidine	1.5
Isoleucine	3.0
Leucine	5.0
Lysine	3.9
Methionine	1.0
Phenylalanine	3.2
Threonine	3.0
Tryptophan	-
Tyrosine	2.2
Valine	3.8
Aspartic Acid	16.3
Glutamic Acid	7.2
Alanine	3.7
Glycine	3.8
Proline	5.6
Serine	3.5

2.1.2 Antihypertensive effect of *Hibiscus Sabdariffa L*.

Aqueous extract of petals exhibited antihypertensive and cardioprotective effects in rats (Odigie *et al*., 2003 a). Infusion of *hibiscus sabdariffa* is also found to lower both systolic and diastolic pressure significantly in spontaneously hypertensive and normotensive rats (Onyenekwe *et al*., 1999). Tea of calyces showed 11.2% reduction in the systolic blood pressure and 10.7% decrease in diastolic pressure (Faraj and Haji, 1999). Effectiveness and tolerability of a standardized extract was studied in patients with mild to moderate hypertension, which revealed a reduction in systolic and diastolic blood pressure by more than 10 percent (Herrera-Allerano *et al*., 2004).

The aqueous extract of the calyx showed a dose-dependent decrease in mean arterial pressure of rats (Adegunloye *et al*., 1996). The extract has a vasodilator effect in the isolated aortic rings of hypertension. These effects are probably mediated through the endothelium-derived nitric oxide-cGMP-relaxant pathway and inhibition of calcium influx into vascular smooth muscle cells (Ajay, 2007). Daily consumption of tea lowers blood pressure in pre and mildly hypertensive adults and may prove an effective component of the dietary changes recommendation for people at risk of developing hypertension (Daniel *et al*., 2008). A standardized extract has shown effective blood pressure lowering activity in hypertensive humans. A recent double-blinded, reference-controlled trial demonstrated significant reduction in blood pressure in the *hibiscus* group, when compared with lisinopril (Ezegwu, 2002).

2.1.3 Hepatoprotective effects of *Hibiscus Sabdariffa L.*

Protective effect of dried flower extracts of *hibiscus sabdariffa* against oxidative stress in rats primary hepatocytes were demonstrated by Tseng *et al*., (1997). Protocatechuic acid, a simple phenolic compound isolated from *H. sabdariffa* showed protective effect against cytotoxicity and genotoxicity of hepatocytes, induced by t-BHP. One of the mechanisms may be associated with its property of scavenging free radicals (Tseng *et al*., 1996).

The extract of its petals protected rats against cadmium-induced liver, prostate and testis lipoperoxidation (Asagba *et al*., 2007). The extract offers hepatoprotection by influencing the levels of lipid peroxidation products and liver marker enzymes in experimental hyperammonaemia, and this could be due to the free-radical scavenging property of natural antioxidants present in the plant (Musthafa *et al*., 2006). The protective effect of aqueous extract and anthocyanins on paracetamol-induced hepatotoxicity in rats has also been reported by Ali *et al*., (2003). Aqueous-ethanol (1:1) extract of the calyx showed a significant decrease in the level of lipid peroxidation in carbon tetrachloride-induced liver damage (Dahiru *et al*., 2003). However, a study showed that prolonged usage of aqueous-methanol extract of the calyces could cause liver injury (Akindahunsi and Olaleye, 2003).

2.1.4 Antihyperlipidaemic effects of *Hibiscus Sabdariffa L.*

Inhibitory effects of the plant extract on LDL oxidation and anti-hyperlipidaemia in fructose and cholesterol-fed rats was demonstrated (Chau *et al*., 2004). It revealed that the extract reduced the level of LDL and the ratio of LDL-cholesterol to HDL-cholesterol. Consumption of dried calyx ethanol extracts reduced lipid profile in rats (Carvajal *et al.*, 2005). Hypocholesterolemic and antioxidant effects of aqueous extracts in

hypercholesterolemic rats are also reported (Hirunpanich *et al*., 2006). Antioxidant effects of the aqueous extracts of dried calyx using rat LDL was investigated, and the study demonstrated protective effect of roselle on LDL oxidation (Hirunpanich *et al*., 2005). Biochemical dynamics and hypocholesterolemic action of the plant demonstrated that its administration induced significant decrease in the activity of serum Glutamic oxaloacetic transaminase (GOT), Glutamic-pyruvate transaminase (GPT), alkaline and acid phosphatase as well as total serum protein. These values nearly returned to the initial levels after 9 weeks of administration (El Saadany, *et al*., 1991). Aqueous extracts of the petals of red and green plant decreases total plasma concentrations in rat indicating cardiovascular protective effects (Olatunji *et al*., 2005).

2.1.5 Antioxidant Activity of *Hibiscus Sabdariffa L*.

The antioxidant and free-radical-scavenging effects of two fractions of the ethanol extract (chloroform soluble fraction and ethyl acetate soluble fraction) obtained from its dried flowers were investigated by Farombi and Fakoya, (2005), and it was found that both fractions scavenge hydrogen peroxide (79-94%) at the dose of 500 µg. Similarly, the extracts showed inhibitory (70-80%) effects on superoxide anions radicals (O_2^-) at a dose of 1000 µg. The antioxidant activities of three varieties using liposome system have also been reported (Christian *et al*., 2006). The methanol and ethyl acetate extracts showed higher COX-1 enzyme inhibition than COX-2inhibition (Christian *et al*., 2006).

2.1.6 Anticancer property of *Hibiscus Sabdariffa L*.

Anthocyanins can cause cancer cell apoptosis, especially in HL-60 cells (Chang, *et al*., 2005). Antioxidant activity of anthocyanins was evaluated by their effects on LDL oxidation in cell free system and anti-apoptotic abilities in RAW264.7 cells (Chang *et al*., 2006). The study showed that anthocyanins of this plant may be used to inhibit LDL oxidation and oxidized LDL-mediated macrophage apoptosis, serving as a chemopreventive agent. Inhibitory effect of protocatechuic acid on tumour promotion in mouse skin demonstrated that protocatechuic acid possessed potential as a cancer chemopreventive agent against tumour promotion (Tseng *et al*., 1997).

2.1.7 Other Pharmacological Activities of *Hibiscus Sabdariffa L*.

Delphinidin 3-sambubioside, a anthocyanin isolated from the dried calyces of *H. sabdariffa* can induce a dose-dependent apoptosis in human leukemia cells (HL-60), characterized by cell morphology, DNA fragmentation, activation of caspase 3, 8 and 9, and inactivation of poly(ADP) ribose polymerase (Hou *et al*., 2005). Ethanol and aqueous extracts of its calyces possess antipyretic activity in experimental animals (Reanmongokol and Itharat, 2007).

Ethanol extract of the plant reduces the extent of cisplatin-induced sperm abnormality in rats (Amin and Hamza, 2006). Inhibition of intestinal motility by methanol, that is, extract in rats showed a significant dose-dependent relaxant effect on rat ileal strip, comparable to the effect shown by nifedipin and papaverine as reference compounds (Salah *et al.,* 2002). Investigation of its antispasmodic potential revealed that aqueous extract of the calyces inhibited the tone of various isolated muscle preparation (Ali *et al.,* 1991).

Effect of *zobo* drink (*H. sabdariffa* water extract) on the pharmacokinetics of acetaminophen in human volunteers was studied and the results showed no statistically significant change in the absorption parameters $t_{1/2}a$, Ka, T_{max}, C_{max} and $AUC_{O\text{-}alpha}$ after the administration of *zobo* (Kolawole and Maduenyi, 2004). Investigation of the anti-inflammatory activity showed that its extract had no effect on rat paw edema, but has inhibitory effect on yeast-induced pyrexia and a significant effect on the hot plate reaction time (Dafallah and Al-Mustafa, 1996). Polysaccharides from its flowers can stimulate the proliferative and differentiation of human keratinocytes, while the neutral polymers were ineffective. Neuropharmacological effects of the aqueous extract of the calyx in rodents revealed that the extract produced a remarkable dose-dependent decrease in spontaneous motor activity in mice and increased the duration of pentobarbital-induced sleep in rats (Amos *et al.,* 2003).

Anticlastogenic effects of aqueous extract of *H. sabdariffa* fruits in bone marrow cells of mice were studied (Adetutu *et al.,* 2004). The results showed that administration of a crude extract led to a significant reduction of micronuclei in polychromatic erythrocytes and combination of *H sabdariffa* and sodium arsenite reduced significantly the frequencies of micronucleated PCEs induced by sodium arsenite (Jonadet *et al.,* 1990).

Studies on *in vitro* enzyme inhibition and *in vivo* cardioprotective activities revealed that a crude hydroalcoholic extract of *H. sabdariffa* had appreciable enzyme-inhibiting activity towards the angiotensin I converting enzyme, attributed to flavones, but weak inhibiting activities towards elastase, trypsin and alpha-chymotrypsin (Jonadet *et al.,* 1990).

Antibacterial activity of gossypetin isolated from *H. sabdariffa* was carried out and results revealed that the activity may be due to polyphenolic nature of the flavonoid gossypetin (Mounnissamy *et al.,* 2002). Investigation on neurotrophic activity of its calyces in mice indicated that the extract of the calyces might prove to be useful memory restorative agents in the treatment of dementia seen in elderly, which may be due to its anti-acetylcholinesterase property (Joshi and Parle, 2006). The haemostatic effect of the leaves was evaluated to confirm its traditional use to arrest bleeding. The extract enhances coagulation of blood, precipitation of some blood clotting factors and decrease the bleeding time (Plotto, 1999).

Tea made from dry calyces of *H. sabdariffa* was given to human and analysed for uric acid and other chemical composition related to urinary stone risk factors. The results suggest the urocosuric effect of the tea in humans (Vitoon *et al.,* 2008).

2.2 CARBAMAZEPINE

Carbamazepine (CBZ) is an anticonvulsant and mood-stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder, as well as trigeminal neuralgia. It is also used off-label for a variety of indications, including attention-deficit hyperactivity disorder, schizophrenia, phantom limb syndrome, complex regional pain syndrome, paroxysmal extreme pain disorder, neuromyotonia, intermittent explosive disorder, border-line personality disorder, myotonia and post-traumatic stress disorder. Like other anticonvulsants, intrauterine exposure is associated with spinal bifida and neurodevelopmental problems (Jentink *et al.,* 2010; Cummings *et al.,* 2011).

2.2.1 History of Carbamazepine

Carbamazepine (CBZ) was discovered by a chemist Walter Schindler at J.R. Geigy, Novartis International AG in Basel, Switzerland in 1953. Schindler then synthesized the drug in 1960, before its antiepileptic properties was discovered (Schindler and Häfilger, 1954). Carbamazepine is an iminostilbene, a dibenzepine derivative that is chemically and pharmacologically-related to tricyclic antidepressant agents (Bazil and Pedley, 2003). It was approved for the management of seizures in 1974, although it had been introduced a decade earlier for the management of trigeminal neuralgia (Mattson *et al.*, 1985). Carbamazepine is a conventional antiepileptic drug, which has efficacy in attenuating picrotoxin-induced convulsion (Ali *et al*., 2003). This may be attributed to its mechanism of action, that is, sodium channel blockade, weak GABAergicn and antiglutamatergic effects (Motohashi, 1992).

2.2.2 Brand Names of Carbamazepine

They are Carbamazepine, Tegretol, Tegretol retard, Carbagen (Erica and Mark, 2010).

2.2.3 Chemistry of Carbamazepine

Its chemical name is 5H-dibenz[b,f]azepine-5-carboxamide, and its structural formula is shown in figure 2.1:

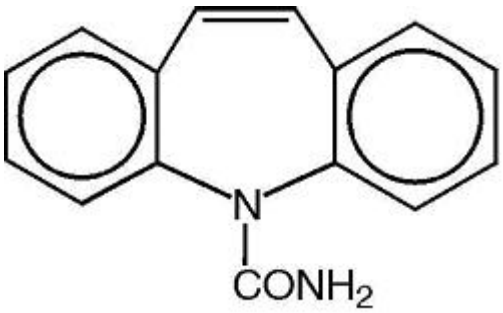


Figure 2.1: Structure of carbamazepine (António *et al*., 2002)

Carbamazepine is available in form of tablets of various strengths, oral liquid containing 100 mg/5ml and suppositories, each containing 125 mg (Erica and Mark, 2010).

2.2.4 Medical Uses of Carbamazepine

Carbamazepine is typically used for the treatment of seizure disorders and neuropathic pain. It may be used as a second line treatment for bipolar disorder and along with antipsychotic agents in schizophrenia. Carbamazepine is the usual drug of choice for patients with newly diagnosed partial onset seizure (Gamble *et al.*, 2009).

2.2.5 Mechanism of Action of Carbamazepine

It acts by stabilizing inactivated sodium channels. Voltage-gated sodium channels which are the molecular pores that allow brain cells (neurones) to generate action potentials - the electrical events that allow neurones to communicate over long distances. After the sodium channels open to start the action potential, they are then inactivated, essentially closing the channels. Carbamazepine will then sustain this closure so that fewer of these channels are available to subsequently open, making brain cells less excitable (Granger *et al.,* 1995). Carbamazepine has also been shown to potentiate GABA receptors, made up of α1, β2, γ2 subunits (Granger *et al.,* 1995).

2.2.6 Pharmacokinetics of Carbamazepine

Once administered, the absorption of CBZ in the body is slow and unpredictable with approximately 2-3% of the applied dose discharged via urine. Studies indicate that CBZ concentration in the blood plasma peaks about 4 to 8 hours after ingestion, but it may take up to 26 hours for the CBZ to take effect (Guneysel *et al*., 2008). Carbamazepine is metabolized by the cytochrome P_{450} system in the liver, producing several metabolites (Guneysel *et al.,* 2008). Carbamazepine exhibits auto-induction; it induces the expression of the hepatic microsomal enzyme system, CYP3A4 which metabolizes carbamazepine itself. Upon initiation of therapy, concentrations are predictable and follow their respective base-line clearance/half-life values that have been established for the specific patient (Bauer, 2008). However, after enough carbamazepine has been presented to the liver, the CYP3A4 activity increases, speeding up its clearance and shortening the half-life. Auto-induction will continue with subsequent increase in dose, but will reach a plateau within 5-7 days of a maintenance dose. Increase in dose of 200 mg every 1-2 weeks may be required to achieve a stable threshold. Stable CBZ plasma concentrations occur within 2-3 weeks after initiation of therapy (Bauer, 2008).

2.2.7 Interactions of Carbamazepine

Carbamazepine has a very high potential for drug interactions. Caution should be taken in combining other medicines with it, including other antiepileptics and mood stabilizers. Lower levels of CBZ are used when co-administered with phenobarbital, phenytoin or primidone. Carbamazepine as a CYP450 inducer may increase clearance of many drugs and decreasing their blood levels. Drugs that are more rapidly metabolized when administered with CBZ include warfarin, phenytoin, theophylline and valproic acid (Lacy, 2009). Drugs

that decrease the metabolism of CBZ or otherwise increase its serum levels include erythromycin (macrolide antibiotic) (Stafstrom *et al*., 1995), cimetidine (H2-receptor blocker), propoxyphene (analgesic) and calcium-channel blockers (such as verapamil). Carbamazepine also increases the metabolism of the hormones in birth control pills, and can reduce their effectiveness leading to unexpected pregnancies (Lacy, 2009).

2.2.8 Adverse Effects of Carbamazepine

The most common dose-related adverse effects of CBZ are headaches and migraines, motor coordination impairment and diplopia. Less common side-effects may include cardiac arrhythmias, blurry or double vision and/or the temporary loss of blood cells, mild digestive upsets and at much higher doses, drowsiness, hyponatraemia and water intoxication have occasionally occurred (Porter and Meldrum, 2007). Carbamazepine has been reported to cause sedation which may be related to the induction of cytochrome P_{450} (Nowakowska, 2011). There are also rare reports of an auditory side-effect for carbamazepine use, whereby patients perceive sounds about a semitone lower than previously (Yoshikawam and Abe, 2003). Thus, middle C would be heard as the note B_3 just below it, and so on. The inverse effect (that is, notes sounding higher) has also been recorded. This unusual side-effect is usually not noticed by most people, and quickly disappears after the person stops taking carbamazepine (Miyaoka *et al*., 2000).

Luszcki, (2004) observed a decrease in locomotor and rearing activities in mice administered CBZ. Carbamazepine has also been linked to serious cognitive anomalies and

eletroencephalogram (EEG) slowing (Salinsky *et al*., 2002). Higher errors of omission following CBZ relative to phenytoin (PHE) administration (Shannon and Love 2005), and more deterioration in memory has been observed, following CBZ administration than repeated dosing with phenytoin (PHE) (Shannon and Love, 2004). Carbamazepine, however, appears to have a more favourable profile than phenytoin and phenobarbital when a study was performed on cognitive effects of AEDs in humans (Ogunrin *et al.*, 2005). Haematological toxicity of CBZ is well documented; CBZ has been reported in an earlier study to cause decrease RBC counts, apparently due to isolated cessation of RBC production, as a result of pure RBC aplasia (Bedangshu *et al*., 2010). However, McNamara, (2006) reported that the prevalence of aplastic anaemia appears to be 1 in 200,000 patients treated with CBZ monotherapy. CBZ has been reported to cause increased platelet counts which was suggested to be of the reactive type since the drug can cause inflammatory reaction, resulting in increased level of interleukin-6. This has been proposed to induce direct stimulation of platelet, which may have a compensatory effect in CBZ-induced thrombocytopenia (Tutor-Crespo *et al*, (2007).

Carbamazepine administration may cause increased lymphocytes; this may arise due to stimulation of formation of epoxides by the activity of cytochrome P450. The epoxides bind covalently with macromolecules and act as hapten to stimulate immunologic actions, hence lymphocytosis (Gerson *et al*., 1983; Spielberg *et al*., 1986). Ashrafi *et al*. (2010) reported a decrease in immunoglobulins A and G following CBZ administration, and this may cause decreased globulin concentration. Increased alkaline phosphatase (ALP) activity following CBZ therapy was thought to be associated with its effect on bone formation, possibly related to increased bone turn-over (Merete *et al*., 2005). Hogan *et al*., (2000) associated adjunctive

CBZ therapy with weight gain. Therefore, a patient undergoing carbamazepine therapy should be carefully monitored, especially for serious adverse reactions, including pure red cell aplasia.

Various clinical and experimental studies involving the effects of CBZ and other AED on male reproduction have been carried out. They have mainly shown hormonal changes and seminal alterations during adult phase, including reduced sperm motility, sperm morphological alterations and decrease in the sperm concentration (Chen *et al*., 1992; Soliman and El-Eguid, 1999; Isojärvi *et al.,* 2004).

2.2.9 Antiepileptic Drugs Effects on Reproductive Endocrine Function

It is well known that epilepsy, AEDs, and the reproductive system have complex interactions. Fertility is lower in both men and women with epilepsy than in the general population. Moreover, reproductive endocrine disorders are more common among patients with epilepsy than among the population in general. These disorders have been attributed both to epilepsy itself and to use of AEDs (Isojarvi *et al*., 2005).

The use of the liver enzyme-inducing AEDs, phenobarbital, phenytoin and carbamazepine increases serum sex hormone-binding globulin (SHBG) concentrations in both men and women with epilepsy. Over time, the increase in serum SHBG levels leads to diminished bioactivity of testosterone and estradiol, which may result in diminished potency in men and menstrual disorders in some women; and, thus, to reduced fertility. Liver enzyme-inducing AEDs also reduce the efficacy of oral contraceptives (Isojarvi *et al*., 2005).

2.2.10 Effect of Carbamazepine on Testosterone

Carbamazepine blocks voltage-dependent sodium and calcium channels, enhance GABA-nergic transmission and/or antagonize glutamate receptors. Similar neurochemical mechanisms are engaged in the interaction of this drug with synthesis of hypothalamic neurohormones such as gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and growth hormone releasing hormone (GHRH). Carbamazepine also affects hormone metabolism by inhibiting or stimulating cytochrome P-450 iso-enzymes. The influence of CBZ and other antiepileptic drugs on hypothalamic-pituitary-gonadal axis appears to be sex-dependent. In males, valproate decreased follicle-stimulating hormone (FSH) and luteinizing hormone (LH), but elevated dehydroepiandrosterone sulfate (DHEAS) concentrations. Carbamazepine decreased testosterone/SHBG ratio, whereas its active metabolite-oxcarbazepine-had no effect on androgens. In females, valproate decreased FSH-stimulated estradiol release and enhanced testosterone level. On the other hand, carbamazepine decreased testosterone level, but enhanced SHBG concentration. It has been reported that carbamazepine, oxcarbazepine or co-administration of CBZ and valproate decreases thyroxine (T_4) level in patients with no effect on thyrotropin (TSH) (Leskiewicz *et al*., 2008).

2.3 VITAMIN E

Vitamin E refers to a group of eight fat-soluble compounds that include both tocopherols and tocotrienols (Brigelius-Flohe and Traber, 1999). There are many different forms of vitamin E, of which γ-tocopherol is the most common in the North American and African

diet. γ-Tocopherol can be found in corn oil, soybean oil, and margarine (Brigelius-Flohe and Traber, 1999).

α-Tocopherol, the most biologically active form of vitamin E, is the second most common form of vitamin E in the North American diet. This variant of vitamin E can be found most abundantly in wheat germ oil, sunflower, and safflower oils. It is a fat-soluble antioxidant that stops the production of reactive oxygen species (ROS) formed, when fat undergoes oxidation (Herrera, 2001).

2.3.1 Functions of Vitamin E

Vitamin E has many biological functions; the antioxidant function being the most important and/or best known (Bell, 1987). Other functions include enzymatic activities, gene expression and neurological function(s). It has also been suggested that the most important function of vitamin E is in cell signaling instead of antioxidant metabolism (Azzi, 2007).

2.3.2 Mechanism of Action of Vitamin E

a) As an antioxidant, vitamin E acts as a peroxyl radical scavenger, preventing the propagation of free radicals in tissues, by reacting with them to form a tocopheryl radical which will then be oxidized by a hydrogen donor (such as vitamin C), and thus return to its reduced state. As it is fat-soluble, vitamin E is incorporated into cell membranes, which protects them from oxidative damage (Traber and Stevens, 2011).

b) As an enzymatic activity regulator, for instance, protein kinase C (PKC), which plays a role in smooth muscle growth, it can be inhibited by α-tocopherol. α-Tocopherol has a stimulatory effect on the dephosphorylation enzyme, protein phosphatase 2A, which in turn, cleaves phosphate groups from PKC, leading to its deactivation, bringing the smooth muscle growth to a halt (Schneider, 2005)

c) Vitamin E has an effect on gene expression. Macrophages rich in cholesterol are found in the atherogenic tissue. Scavenger receptor CD36 is a class B scavenger receptor found to be up-regulated by oxidized LDL and binds it (Devaraj *et al*., 2001). Treatment with alpha-tocopherol was found to down-regulate the expression of the CD36 scavenger receptor gene and the scavenger receptor class A (SR-A) (Devaraj *et al*., 2001), and modulates expression of the connective tissue growth factor (CTGF) (Villacorta *et al*., 2003). CTGF gene, when expressed, is responsible for the repair of wounds and regeneration of the extracellular tissue that is lost or damaged during atherosclerosis (Villacorta, *et al*., 2003).

d) Vitamin E plays a role in neurological functions and inhibition of platelet aggregation. (Muller, 2010).

e) Vitamin E protects lipids and prevents the oxidation of polyunsaturated fatty acids (PUFAs) (Muller, 2010).

2.3.3 Recommended Daily Intake of Vitamin E

The Food and Nutrition Board at the Institute of Medicine of the U.S. National Academy of Sciences report the following dietary reference intakes for vitamin E in Table 2.5 (Food and Nutrition Board, 2000):

Table 2.5: Dietary intakes for vitamin E at different ages of life (Food and Nutrition Board, 2000)

Mg/day	Age
Infants	
5	0 to 6 months
6	7 to 12 months
Children	
6	1 to 3 years
7	4 to 8 years
11	9 to 13 years
Adolescents and Adult	
15	14 and older

2.3.4 History of Vitamin E

The first use for vitamin E as a therapeutic agent was conducted in 1938 by Widenbauer. Widenbauer used wheat germ oil supplement on 17 premature newborn infants suffering from growth failure. Eleven out of the original 17 patients recovered and were able to resume normal growth rates (Bell, 1987). Later on, in 1948, while conducting experiments on alloxan effects on rats, Gyorge and Rose noted that the rats receiving tocopherol supplements suffered from less haemolysis than those that did not receive tocopherol (Gyorgy, 1948). In 1949, Gerloczy administered all-rac-α-tocopheryl acetate to prevent and cure edema (Gerloczy, 1949; Brion *et al*., 2003). The methods of administration used were both oral, which showed positive response; and intramuscular, which did not show a response (Bell, 1987). This early investigative work on the benefits of vitamin E supplementation was the gateway to curing the vitamin E deficiency induced haemolytic anaemia described during the 1960s. Since then, supplementation of infant formulas with vitamin E has eradicated this vitamin deficiency as a cause for hemolytic anemia (Bell, 1987).

2.3.5 Forms of Vitamin E

The eight forms of vitamin E are divided into two groups; four are tocopherols and four are tocotrienols. They are identified by prefixes α-, β-, γ-, and δ-. Natural tocopherols occur in the RRR-configuration only. The synthetic form contains eight different stereoisomers and is called *all-rac*-α-tocopherol (Traber and Stevens, 2011).

2.3.5.1 α-Tocopherol

α-Tocopherol is an important lipid-soluble antioxidant. It performs its functions as antioxidant in what is known by the glutathione peroxidase pathway (Traber and Stevens, 2011). It protects cell membranes from oxidation by reacting with lipid radicals, produced in the lipid peroxidation chain reaction (Herrera, 2001). This would remove the free-radical intermediates and prevent the oxidation reaction from continuing. The oxidized α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Wang, 1999). However, the importance of the antioxidant properties of this molecule at the concentrations present in the body are not clear and it is possible that the reason why vitamin E is required in the diet is unrelated to its ability to act as an antioxidant (Brigelius-Flohé, 2009). Other forms of vitamin E have their own unique properties; for example, gamma-tocopherol is a nucleophile that can react with electrophilic mutagens (Brigelius-Flohe, 1999).

2.3.5.2 Tocotrienols

Compared with tocopherols, tocotrienols are sparsely studied (Traber, 1996; Sen and Roy, 2006). Less than 1% of Public Medicine papers published on vitamin E relate to tocotrienols (Sen and Roy, 2006). The current research direction is starting to give more prominence to the tocotrienols, the lesser known but more potent antioxidants in the vitamin E family. Some studies have suggested that tocotrienols have specialized roles in protecting neurones from damage (Sen and Roy, 2006), and cholesterol reduction by inhibiting the activity of HMG-CoA reductase. Delta-tocotrienol blocks the processing of sterol regulatory element-binding proteins (SREBPs) (Das *et al*., 2008).

2.4 ANTIOXIDANTS

Antioxidants are molecules capable of slowing or preventing the oxidation of other damaging oxidants (Halliwell and Gutteridge, 2007). Based on function, antioxidants have been divided into four groups; the first line of defence is the prevention of oxidants which are involved in the formation of free radicals. The second line of defence is the radical scavenging antioxidants; the third line of defence is the repair and *de novo* antioxidants, while the fourth is adaptive in nature (Noguchi and Niki, 1999).

Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Oxidative stress is damage to cell structure and cell function by overly reactive oxygen-containing molecules and chronic excessive inflammation. Oxidative stress seems to play a significant role in many human diseases, including cancers. The use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. For these reasons, oxidative stress can be considered to be both the cause and the consequence of some diseases (Prabhat *et al*., 1995).

Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as altitude sickness, cancer, and even coronary heart disease. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials with a limited number of antioxidants detected no benefit and even

suggested that excess supplementation with certain putative antioxidants may be harmful (Baillie *et al.,* 2009).

2.4.1 Antioxidant System and Oxidative Stress in the Testes

Spermatogenesis is an extremely active replication process capable of generating approximately 1, 000 sperms a second. The high rates of cell division inherent in this process simply correspondingly high rates of mitochondrial oxygen consumption by the germ cell epithelium. However, the poor vascularization of the testes means that oxygen tensions in the testicular tissues are low and that the competition for the vital element within the testes is extremely intense (Aitken and Shaun, 2008). Since both spermatogenesis and Leydig cell steroidogensis are vulnerable to oxidative stress, the low oxygen tension that characterizes this tissue may be an important component of the mechanism by which the testes protect itself from free-radical-mediated damage. In addition the testes contain an elaborate array of antioxidant enzymes and free-radical scavengers to ensure that the twin spermatogenesis and steroidogenic functions of the testes are not impacted by oxidative stress (Chen *et al*., 2005).

The antioxidant defence systems are of major importance because peroxidative damage is currently regard as single most important cause of impaired testicular functions underpinning the pathological consequence of a wide range of conditions from testicular torsion to diabetes and xenobiotic exposure. Despite the low oxygen tensions that characterize the testicular micro-environment, the testicular tissue remains vulnerable to oxidative stress due to abundance of highly unsaturated fatty acids and the presence of potential ROS-generating system. Reactive oxygen species can be from mitochondria and a

variety of enzymes including the xanthine- and NADPH-oxidases, and the cytochrome P_{450s} (Banfi *et al*., 2001; Kumagai *et al*., 2002). the induction of oxidative stress in the testes precipitate a response characterized by the NF-kB mediated induction of mRNA species for superoxide dismutase, glutathione peroxidase, glutathione-s-transferase and catalase activity (Kanr *et al*., 2006).

Mouse testes contain two distinct superoxide dismutase (SOD-1) transcripts which differ by 114 nucleotides in their 5' untranslated regions (UTRs). The shorter SOD-1 mRNA, a somatic type SOD-I mRNA (SSOD-1), is ubiquitously expressed in all somatic tissues as well as in testes. The larger SOD-1 mRNA, a testis-specific SOD-1 mRNA (TSOD-1), derived from an alternative upstream start site, is transcribed solely in postmeiotic germ cells and is translationally regulated during spermiogenesis (Gu and Hecht, 1996).

2.4.2 Mechanism of Action of Antioxidants

As a first line of defence against agents which have the ability to cause oxidative stress, the cell is equipped with inherent antioxidant network. Antioxidants are capable of donating electrons to oxidants, thus terminating their reactivity, in this process; the antioxidants are converted into radicals without the ability to cause damage due to their stable state (Lykkesfeldt *et al*., 2003). The second line of defense involves the ability of the body to detect and repair or remove oxidized and damaged molecules. DNA repairing enzymes capable of detecting oxidized bases or misinterpretations, cutting them out and inserting the correct undamaged base DNA are involved in this process. The third line of defence involves the use of suicidal apoptosis (Payne *et al*., 1995). This occurs when the extent of oxidative damage is high and the capacity to repair and remove damaged molecules is overwhelmed.

2.4.3 Enzyme systems

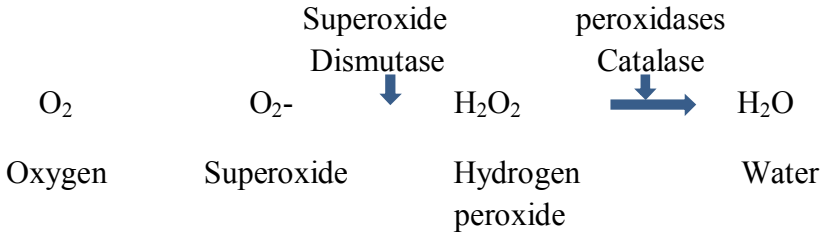


Figure 2.2: Enzymatic pathway for detoxification of reactive oxygen species (Carr and Frei, 1999)

As with the chemical antioxidants, cells are protected against oxidative stress by an interacting network of antioxidant enzymes. Here, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutases catalysing the first step and then catalases and various peroxidases removing hydrogen peroxide (fig. 2.2). As with antioxidant metabolites, the contributions of these enzymes to antioxidant defences can be hard to separate from one another, but the generation of transgenic mice lacking just one antioxidant enzyme can be informative (Ho *et al*., 1998).

2.4.4 Classification of Antioxidants

2.4.4.1 Natural antioxidants: Natural occurring antioxidants of high or low molecular weight can differ in their composition, physical and chemical properties, mechanism and site of action. They can be divided into the following categories:

a) **Enzymes:** Enzyme such as superoxide dismutase (SOD), catalase and glutathione peroxidase attenuate the generation of ROS by removing potential oxidants or by transferring ROS/RNS (reactive nitrogen species) into relatively stable compounds.

Superoxide dismutase which was discovered in late 60s, catalyzes the transformation of the superoxide radical into hydrogen peroxide, which can then be transformed by enzyme catalase into water and molecular oxygen. While superoxide anion in itself is not particularly reactive, it can reduce transition metal ions, such as iron, and gets converted to the most reactive radical – the hydroxyl radical. Thus, elimination of superoxide radical can attenuate the formation of hydroxyl radical. Glutathione peroxidase (GPx) reduces lipid peroxides (ROOH), formed by the oxidation of PUFA, to a stable, non-toxic molecule – hydroxyl fatty acid (ROH). Together with phospholipase, GPx can also convert phospholipids hydroperoxide (PL-OOH) into phospholipids hydroxide (PL-OH) (Ursini *et al*., 1982).

b) **Low-molecular weight antioxidants:** These are subdivided into lipid-soluble antioxidants (tocopherol, carotenoids, quinones, bilirubin and some polyphenols) and water soluble antioxidants (ascorbic acid, uric acid and polyphenols). These delay or inhibit cellular damage mainly through free-radical-scavenging property (Ursini *et al*., 1982).

c) **Lipid soluble antioxidants**: These antioxidants tend to accumulate in lipid plasma lipoprotein (for example, LDL); upon supplementation. This group of antioxidants is supposed to act as highly efficient scavengers, such as against lipid peroxyl radical, which are formed within the lipoprotein as a consequence of free-radical chain reaction of lipid peroxidation (Ursini *et al*., 1982).

d) **Water soluble antioxidants:** These antioxidants cannot enter the lipid moiety of low density lipoprotein (LDL). These are less efficient as they are principally unable to encounter most of these lyophilic radical. However, such compounds may act in a

synergistic manner with lipophilic antioxidants by regenerating them (Ursini *et al*., 1982.).

e) **Synthetic antioxidants:** These are most effective antioxidants and are synthetic chemicals, approved by food and drug administration for addition to foods; for example, Butylated hydroxyl anisole, Butylatedhydroxy toluene and Tertiary butylatedhydroxyquinone etc.

2.4.5. Mode of action of antioxidants

In general, the antioxidants act by the following mechanisms:

a) Chain-breaking reaction; for example, α-tocopherol, which act in a lipid phase to trap free radicals.
b) By reducing concentration of reactive oxygen species e.g. glutathione.
c) By scavenging initiating radicals for example, SOD, which acts in the lipid phase to trap superoxide free radicals.
d) By chelating transition metal catalyst. This is a group of compounds, which act by sequestration of transition metals that are well established peroxidants. In this way transferring, lactoferrin and ferritin function to keep iron-induced oxidant stress in check, while ceruloplasmin and albumin are copper sequestrants (Ursini *et al*., 1982).

2.4.6 Types of Antioxidants

2.4.6.1 Uric acid

Uric acid is by far the highest concentration antioxidant in human blood. Uric acid (UA) is an antioxidant oxypurine produced from xanthine by the enzyme xanthine oxidase, and is an intermediate product of purine metabolism. In almost all land animals, urate oxidase further catalyzes the oxidation of uric acid to allantoin; but in humans and higher primates, the urate oxidase gene is non-functional, so that UA is not further broken down. The evolutionary reasons for this loss of urate converstion to allantoin remain the topic of active speculation. The antioxidant effects of UA have led researchers to suggest that this mutation was beneficial to early primates and humans. Studies of high altitude acclimatization support the hypothesis that urate acts as an antioxidant by mitigating the oxidative stress caused by high-altitude hypoxia. With respect to multiple sclerosis (MS), Gwen Scott, (2002) explains the significance of UA as an antioxidant by proposing that "Serum UA levels are inversely associated with the incidence of MS in humans because MS patients have low serum UA levels and individuals with hyperuricaemia (gout) rarely develop the disease. Moreover, the administration of UA is therapeutic in experimental allergic encephalomyelitis, an animal model of MS. In summary, while the mechanism of UA as an antioxidant is well-supported, the claim that its levels affect MS risk is still controversial (Massa *et al*., 2009).

Likewise, UA has the highest concentration of any blood antioxidant and provides over half of the total antioxidant capacity of human serum. Uric acid's antioxidant activities are also complex, given that it does not react with some oxidants, such as superoxide, but does act against peroxynitrite, peroxides, and hypochlorous acid. Concerns over elevated UA's

contribution to gout must be considered as one of many risk factors. By itself, UA-related risk of gout at high levels (415–530 μmol/L) is only 0.5% per year with an increase to 4.5% per year at UA supersaturation levels (535+ μmol/L). Studies by Gwen Scott, (2002) determined UA's antioxidant actions within normal physiological levels, and found antioxidant activity at levels as high as 285 μmol/L (Nazarewicz *et al*., 2007).

2.4.6.2 Ascorbic acid (Vitamin C)

Ascorbic acid, or vitamin C, is a monosaccharide oxidation-reduction (redox) catalyst found in both animals and plants. Humans must obtain it from the diet. Most other animals are able to produce this compound in their bodies and do not require it in their diets. Ascorbic acid is required for the conversion of the procollagen to collagen by oxidizing proline residues to hydroxyproline. In other cells, it is maintained in its reduced form by reaction with glutathione, which can be catalysed by protein disulfide isomerase and glutaredoxins. Ascorbic acid is a redox catalyst which can reduce, and thereby neutralize, ROS such as hydrogen peroxide. In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the redox enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants. Ascorbic acid is present at high levels in all parts of plants and can reach concentrations of 20 millimolar in chloroplasts (Smirnoff *et al*., 2000; Shigeoka *et al*., 2002).

2.4.6.3 Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is not required in the diet and is instead synthesized in cells from its constituent amino acids.

Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems, such as ascorbate in the glutathione-ascorbate cycle, glutathione peroxidases and glutaredoxins, as well as reacting directly with oxidants. Due to its high concentration and its central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants. In some organisms glutathione is replaced by other thiols, such as by mycothiol in the actinomycetes, bacillithiol in some Gram-positive bacteria, or by trypanothione in the kinetoplastids (Fahey, 2001; Gaball *et al*., 2010).

2.4.6.4 Melatonin

Melatonin is a powerful antioxidant. Melatonin easily crosses cell membranes and the blood–brain barrier. Unlike other antioxidants, melatonin does not undergo redox cycling, which is the ability of a molecule to undergo repeated reduction and oxidation. Redox cycling may allow other antioxidants (such as vitamin C) to act as pro-oxidants and promote free radical formation. Melatonin, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Tan *et al*., 2000).

2.4.6.5 Superoxide dismutase

Superoxide dismutases (SODs) are a class of closely-related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide. SOD enzymes are present in almost all aerobic cells and in extracellular fluids. Superoxide dismutase enzymes

contain metal ion co-factors that, depending on the isozyme, can be copper, zinc, manganese or iron. In humans, the copper/zinc SOD is present in the cytosol, while manganese SOD is present in the mitochondrion. There is also a third form of SOD in extracellular fluids, which contains copper and zinc in its active sites. The mitochondrial isozyme seems to be the most biologically important of these three, since mice lacking this enzyme die soon after birth. In contrast, the mice lacking copper/zinc SOD (SOD1) are viable but have numerous pathologies and a reduced lifespan, while mice without the extracellular SOD have minimal defects (sensitive to hyperoxia) (Van Camp *et al*., 1997).

2.4.6.6 Catalase

Catalases are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor. This protein is localized to peroxisomes in most eukaryotic cells. Catalase is an unusual enzyme since, although hydrogen peroxide is its only substrate, it follows a ping-pong mechanism. Here, its co-factor is oxidised by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate. Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase — acatalasemia — or mice genetically engineered to lack catalase completely, suffer few ill effects (Mueller *et al*., 1997).

2.4.6.7 Peroxiredoxins

Peroxiredoxins are peroxidases that catalyze the reduction of hydrogen peroxide, organic hydroperoxides, as well as peroxynitrite. They are divided into three classes: typical 2-

cysteine peroxiredoxins; atypical 2-cysteine peroxiredoxins; and 1-cysteine peroxiredoxins. These enzymes share the same basic catalytic mechanism, in which a redox-active cysteine (the peroxidatic cysteine) in the active site is oxidized to a sulfonic acid by the peroxide substrate. Over-oxidation of this cysteine residue in peroxiredoxins inactivates these enzymes, but this can be reversed by the action of sulfiredoxin. Peroxiredoxins seem to be important in antioxidant metabolism, as mice lacking peroxiredoxin 1 or 2 have shortened life-span and suffer from haemolytic anaemia, while plants use peroxiredoxins to remove hydrogen peroxide generated in chloroplasts (Lee *et al*., 2003; Dietz *et al*., 2006).

2.4.7 Pro-Oxidant Activities of Antioxidant

Antioxidants that are reducing agents can also act as pro-oxidants. For example, vitamin C has antioxidant activity when it reduces oxidizing substances such as hydrogen peroxide; however, it will also reduce metal ions that generate free radicals through the Fenton reaction (Carr and Frei, 1999).

$2\ Fe^{3+} + \text{Ascorbate} \rightarrow 2\ Fe^{2+} + \text{Dehydroascorbate}$

$2\ Fe^{2+} + 2\ H_2O_2 \rightarrow 2\ Fe^{3+} + 2\ OH\bullet + 2\ OH^-$

The relative importance of the antioxidant and pro-oxidant activities of antioxidants are an area of current research, but vitamin C, which exerts its effects as a vitamin by oxidizing polypeptides, appears to have a mostly antioxidant action in the human body. However, less data are available for other dietary antioxidants, such as vitamin E, or the polyphenols. Likewise, the pathogenesis of diseases involving hyperuricemia likely involves direct and

indirect pro-oxidant properties of uric acid (Schneider, 2005). That is, paradoxically, agents which are normally considered antioxidants can act as conditional pro-oxidants and actually increase oxidative stress. Besides ascorbate, medically important conditional pro-oxidants include uric acid and sulfhydryl amino acids such as homocysteine. Typically, this involves some transition-series metal such as copper or iron as catalyst (Halliwell, 2008).

2.4.8 Potential Health Effects of Antioxidants

The brain is uniquely vulnerable to oxidative injury, due to its high metabolic rate and elevated levels of polyunsaturated lipids, the target of lipid peroxidation (Reiter, 1995). Consequently, antioxidants are commonly used as medications to treat various forms of brain injury. Some antioxidants like superoxide dismutase, mimetics, sodium thiopental and propofol are used to treat reperfusion injury and traumatic brain injury. These compounds appear to prevent oxidative stress in neurones and prevent apoptosis and neurological damage. Antioxidants are also being investigated as possible treatments for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, and as a way to prevent noise-induced hearing loss. Targeted antioxidants may lead to better medicinal effects. Mitochondria-targeted ubiquinone, for example, may prevent damage to the liver caused by excessive alcohol (Di Matteo and Esposito 2003; Warner *et al*., 2004).

People who eat fruits and vegetables have a lower risk of heart disease and some neurological diseases, and there is evidence that some types of vegetables, and fruits in general, may lower risk against some cancers. Since fruits and vegetables happen to be good sources of nutrients and phytochemicals, this suggests that antioxidant compounds might

lower risk against several diseases. This idea has been tested in a limited manner in clinical trials and does not seem to be true, as antioxidant supplements have no clear effect on the risk of chronic diseases such as cancer and heart disease. This suggests that these health benefits come from other substances in fruits and vegetables (possibly dietary fibre) or come from a complex mixture of compounds. For example, the antioxidant effect of flavonoid-rich foods seems to be due to fructose-induced increases in the synthesis of the antioxidant uric acid and not to dietary antioxidants (Lotito and Frei, 2006).

Several trials have investigated supplementation with high doses of antioxidants. Among these studies is "Supplémentation en Vitamines et Mineraux Antioxydants" (SU.VI.MAX) study that tested the effect of supplementation with doses comparable to those in a healthy diet. Over 12,500 French men and women took either low-dose antioxidants (120 mg of ascorbic acid, 30 mg of vitamin E, 6 mg of beta carotene, 100 μg of selenium, and 20 mg of zinc) or placebo pills for an average of 7.5 years. The study concluded that low-dose antioxidant supplementation lowered total cancer incidence and all-cause mortality in men, but not in women. Supplementation may be effective in men only because of their lower baseline status of certain antioxidants, especially of beta carotene (Hercberg *et al*., 2004).

Although some levels of antioxidant vitamins and minerals in the diet are required for good health, there is considerable doubt as to whether antioxidant supplements are beneficial or harmful; and if they are actually beneficial, which antioxidant(s) are needed and in what amounts. Indeed, some authors argue that the hypothesis that antioxidants could prevent chronic diseases has now been disproved and that the idea was misguided from the beginning. Rather, dietary polyphenols may have non-antioxidant roles in minute

concentrations that affect cell-to-cell signaling, receptor sensitivity, inflammatory enzyme activity or gene regulation (Williams *et al*., 2004; Virgili, and Marino, 2008).

For overall life expectancy, it has even been suggested that moderate levels of oxidative stress may increase lifespan in the worm *Caenorhabditis elegans*, by inducing a protective response to increased levels of reactive oxygen species. The suggestion that increased life expectancy comes from increased oxidative stress conflicts with results seen in the yeast *Saccharomyces cerevisiae*, and the situation in mammals is even less clear (Green, 2008).

2.4.9 Effect of Antioxidant on Physical Exercise

During exercise, oxygen consumption can increase by a factor of more than 10. This leads to a large increase in the production of oxidants and results in damage that contributes to muscular fatigue during and after exercise. The inflammatory response that occurs after strenuous exercise is also associated with oxidative stress, especially in the 24 hours after an exercise session. The immune system response to the damage done by exercise peaks 2 to 7 days after exercise, which is the period during which most of the adaptation that leads to greater fitness, occurs. During this process, free radicals are produced by neutrophils to remove damaged tissue. As a result, excessive antioxidant levels may inhibit recovery and adaptation mechanisms. Antioxidant supplements may also prevent some of the health gains that normally come from exercise, such as increased insulin sensitivity (Ristow *et al*., 2009). The evidence for benefits from antioxidant supplementation in vigorous exercise is mixed. There is strong evidence that one of the adaptations resulting from exercise is a strengthening of the body's antioxidant defences, particularly the glutathione system, to regulate the increased oxidative stress. This effect may be to some extent protective against

diseases which are associated with oxidative stress, which would provide a partial explanation for the lower incidence of major diseases and better health of those who undertake regular exercise. No benefits for physical performance to athletes are seen with vitamin E supplementation. Indeed, despite its key role in preventing lipid membrane peroxidation, 6 weeks of vitamin E supplementation had no effect on muscle damage in ultramarathon runners. Although there appears to be no increased requirement for vitamin C in athletes, there is some evidence that vitamin C supplementation increased the amount of intense exercise that can be done and vitamin C supplementation before strenuous exercise may reduce the amount of muscle damage. Other studies found no such effects, and some research suggests that supplementation with amounts as high as 1000 mg inhibits recovery (Ristow *et al*., 2009).

2.4.10 Adverse Effects of Antioxidants

Relatively strong reducing acids can have anti-nutrient effects by binding to dietary minerals such as iron and zinc in the gastrointestinal tract and preventing them from being absorbed. Notable examples are oxalic acid, tannins and phytic acid, which are high in plant-based diets. Calcium and iron deficiencies are not uncommon in diets in developing countries where less meat is eaten and there is high consumption of phytic acid from beans and unleavened whole grain bread (Gibson *et al*., 2006).

Non-polar antioxidants such as eugenol a major component of oil of cloves have toxicity limits that can be exceeded with the misuse of undiluted essential oils. Toxicity associated with high doses of water-soluble antioxidants such as ascorbic acid are less of a concern, as these compounds can be excreted rapidly in urine. More seriously, very high doses of some

antioxidants may have harmful long-term effects. The beta-Carotene and Retinol Efficacy Trial (CARET) study of lung cancer patients found that smokers given supplements containing beta-carotene and vitamin A had increased rates of lung cancer. Subsequent studies confirmed these adverse effects. These harmful effects may also be seen in non-smokers, as a recent meta-analysis including data from approximately 230,000 patients showed that β-carotene, vitamin A or vitamin E supplementation is associated with increased mortality but saw no significant effect from vitamin C (Block *et al*., 2008).

No health risk was seen when all the randomized controlled studies were examined together, but an increase in mortality was detected only when the high-quality and low-bias risk trials were examined separately. As the majority of these low-bias trials dealt with either elderly people, or people already suffering disease, these results may not apply to the general population. While antioxidant supplementation is widely used in attempts to prevent the development of cancer, antioxidants may interfere with cancer treatments, since the environment of cancer cells causes high levels of oxidative stress, making these cells more susceptible to the further oxidative stress induced by treatments. As a result, by reducing the redox stress in cancer cells, antioxidant supplements could decrease the effectiveness of radiotherapy and chemotherapy. On the other hand, other reviews have suggested that antioxidants could reduce side effects or increase survival times (Albanes, 1999; Block *et al*., 2008).

2.4.11 Antioxidant Use in Food Preservation Technology

Antioxidants are used as food additives to help guard against food deterioration. Exposure to oxygen and sunlight are the two main factors in the oxidation of food, so food is preserved

by keeping in the dark and sealing it in containers or even coating it in wax, as with cucumbers. However, as oxygen is also important for plant respiration, storing plant materials in anaerobic conditions produces unpleasant flavors and unappealing colours. Consequently, packaging of fresh fruits and vegetables contains an ~8% oxygen atmosphere. Antioxidants are an especially important class of preservatives as, unlike bacterial or fungal spoilage, oxidation reactions still occur relatively rapidly in frozen or refrigerated food. These preservatives include natural antioxidants such as ascorbic acid (AA, E300) and tocopherols (E306), as well as synthetic antioxidants such as propyl gallate (PG, E310), tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA, E320) and butylated hydroxytoluene (BHT, E321) (Iverson, 1995).

The most common molecules attacked by oxidation are unsaturated fats; oxidation causes them to turn rancid. Since oxidized lipids are often discolored and usually have unpleasant tastes such as metallic or sulfurous flavors, it is important to avoid oxidation in fat-rich foods. Thus, these foods are rarely preserved by drying; instead, they are preserved by smoking, salting or fermenting. Even less fatty foods such as fruits are sprayed with sulfurous antioxidants prior to air drying. Oxidation is often catalyzed by metals, which is why fats such as butter should never be wrapped in aluminium foil or kept in metal containers. Some fatty foods such as olive oil are partially protected from oxidation by their natural content of antioxidants, but remain sensitive to photo-oxidation. Antioxidant preservatives are also added to fat-based cosmetics such as lipstick and moisturizers to prevent rancidity (Del Carlo *et al*., 2004).

2.4.12 Measurement and Levels of Antioxidant in Food

Measurement of antioxidants is not a straightforward process, as this is a diverse group of compounds with different reactivities to different reactive oxygen species. In food science, the oxygen radical absorbance capacity (ORAC) used to be the industry standard for antioxidant strength of whole foods, juices and food additives. However, the United States Department of Agriculture (USDA) withdrew these ratings in 2012 as biologically invalid, stating that no physiological proof *in vivo* existed to support the free-radical theory. Consequently, the ORAC method, derived only in *in vitro* experiments, is no longer considered relevant to human diets or biology. Alternative *in vitro* measurements include the Folin-Ciocalteu reagent, and the Trolox equivalent antioxidant capacity assay (Prior *et al*., 2005).

Antioxidants are found in vegetables, fruits, grain cereals, eggs, meat, legumes and nuts as shown in Table 2.6. Some, such as lycopene and ascorbic acid, can be destroyed by long-term storage or prolonged cooking. Other antioxidant compounds are more stable, such as the polyphenolic antioxidants in foods such as whole-wheat cereals and tea. The effects of cooking and food processing are complex, as these processes can also increase the bioavailability of antioxidants, such as some carotenoids in vegetables. Processed food contains fewer antioxidants than fresh and uncooked foods, as preparation exposes food to oxygen (Henry and Heppell, 2002).

Table 2.6: Food containing antioxidant compound (Beecher, 2003)

Antioxidant compounds	**Foods containing high levels of these antioxidants**

Vitamin C (ascorbic acid)	Fresh Fruits and vegetables
Vitamin E (tocopherols, Tocotrienols)	Vegetable oils
Polyphenolic antioxidants (resveratrol, flavonoids)	Tea, coffee, soy, fruit, olive oil, chocolate, cinnamon, oregano
Carotenoids (lycopene, carotenes, lutein)	Fruit, vegetables and eggs

2.4.13 Non-vitamin Endogenous Anti-Oxidants

Other antioxidants are not vitamins and are instead made in the body. For example, ubiquinol (coenzyme Q) is poorly absorbed from the gut and is made in humans through the mevalonate pathway. Another example is glutathione, which is made from amino acids. As any glutathione in the gut is broken down to free cysteine, glycine and glutamic acid before being absorbed, even large oral doses have little effect on the concentration of glutathione in the body. Although large amounts of sulfur-containing amino acids such as acetylcysteine can increase glutathione, no evidence exists that eating high levels of these glutathione precursors is beneficial for healthy adults. Supplying more of these precursors may be useful as part of the treatment of some diseases, such as acute respiratory distress syndrome, protein-energy malnutrition, or preventing the liver damage produced by paracetamol overdose (Dodd *et al.*, 2008).

Other compounds in diet can alter the levels of antioxidants by acting as pro-oxidants. Here, consuming the compound causes oxidative stress, which the body responds to by inducing higher levels of antioxidant defenses such as antioxidant enzymes. Some of these

compounds, such as isothiocyanates and curcumin, may be chemopreventive agents that either block the transformation of abnormal cells into cancerous cells, or even kill existing cancer cells (Pan and Ho, 2008).

2.5 SEMINAL FLUID

Semen, also known as seminal fluid, is an organic fluid that may contain spermatozoa. It is secreted by the gonads (sexual glands) and other sexual organs of male or hermaphroditic animals and can fertilize female ova. In humans, seminal fluid contains several components besides spermatozoa known as seminal plasma, proteolytic and other enzymes as well as fructose as elements of seminal fluid which promote the survival of spermatozoa, and provide a medium through which they can move or "swim" (Owen and Katz, 2005).

The seminal fluid also provides a nutritive and protective medium for the spermatozoa during their journey through the female reproductive tract. The normal environment of the vagina is a hostile one for sperm cells, as it is very acidic (from the native microflora producing lactic acid), viscous, and patrolled by immune cells. The components in the seminal plasma attempt to compensate for this hostile environment. Basic amines such as putrescine, spermine, spermidine and cadaverine are responsible for the smell and flavor of semen. These alkaline bases counteract and buffer the acidic environment of the vaginal canal, and protect DNA inside the sperm from acidic denaturation (Owen and Katz, 2005).

2.5.1 Composition of Human Semen

Seminal fluid of humans contains a complex range of organic and inorganic constituents and sperm cells. In the human male, sperm cells are produced by the testes they constitute only

about 2 to 5 percent of the total semen volume. After emerging from the testes, sperm are stored in the epididymis, in which secretions of potassium, sodium, and glycerylphosphorylcholine (an energy source for sperm) are contributed to the sperm cells (Owen and Katz, 2005).

Sperm cell mature in the epididymis; they then pass through a long tube called the ductus, or vas, deferens to another storage area, the ampulla. The ampulla secretes a yellowish fluid, ergothioneine, a substance that reduces (removes oxygen from) chemical compounds. During the process of ejaculation, sperm passes through the ejaculatory ducts and mixes with fluids from the seminal vesicles, the prostate, and the bulbo-urethral glands to form the semen as shown in Figure 2.3 and the percentage contribution are shown in Table 2.7. The seminal vesicles produce a yellowish viscous fluid rich in fructose and other substances like amino acids, citric acid, phosphorus, potassium, and hormones known as prostaglandins that makes up about 60-70% of human semen. The prostatic secretion about 30 percent, influenced by dihydrotestosterone, is a whitish (sometimes clear), thin fluid containing proteolytic enzymes, citric acid, acid phosphatase and lipids. The bulbourethral glands secrete a clear secretion into the lumen of the urethra to lubricate it. Sertoli cells, which nurture and support developing spermatocytes, secrete a fluid into seminiferous tubules that helps transport sperm to the genital ducts. The ductuli efferentes possess cuboidal cells with microvilli and lysosomal granules that modify the ductal fluid by reabsorbing some fluid. Once the semen enters the ductus epididymis the principle cells, which contain pinocytotic vessels indicating fluid reabsorption, secrete glycerophosphocholine which most likely inhibits premature capacitation (Owen and Katz, 2005).

Figure 2.3: Structures involved in the production and transport of semen (*Encyclopædia Britannica,* 2012*)*

Table 2.7: Showing semen components and contributions of reproductive gland (Owen and Katz, 2005)

Gland	Approximate	Description
Testes	2–5%	Approximately 200- to 500-million spermatozoa (also called sperm or spermatozoans), produced in the testes, are released per ejaculation. If a man has undergone a vasectomy, he will have no sperm in ejaculation
Seminal vesicle	60–70%	Amino acids, citrate, enzymes, flavins, fructose (2–5 mg per mL semen, the main energy source of sperm cells, which rely entirely on sugars from the seminal plasma for energy), phosphorylcholine, prostaglandins (involved in suppressing an immune response by the female against the foreign semen), proteins, vitamin C
Prostate gland	25–30%	Acid phosphatase, citric acid, fibrinolysin, prostate specific antigen, proteolytic enzymes, Zinc (the zinc level is about 135±40 micrograms/ml for healthy men, zinc helps to stabilize the DNA-containing chromatin in the sperm cells; zinc deficiency may result in lowered fertility because of increased sperm fragility, zinc deficiency can also adversely affect spermatogenesis)
Bulbour-ethral gland	25–30%	Galactose, mucus (serve to increase the mobility of sperm cells in the vagina and cervix by creating a less viscous channel for the sperm cells to swim through, and preventing their diffusion out of the semen; contributes to the cohesive jelly-like texture of semen), pre-ejaculate fluid, sialic acid

A 1992 World Health Organization report described normal human semen as having a volume of 2 ml or greater, pH of 7.2 to 8.0, sperm concentration of 20×10^6 spermatozoa/ml or more, sperm count of 40×10^6 spermatozoa per ejaculate or more, and motility of 50% or more with forward progression (categories a and b) of 25% or more with rapid progression (category a) within 60 minutes of ejaculation (World Health Organization, 2003).

A 2005 review of the literature found that the average reported physical and chemical properties of human semen were as follows in Table 2.8: (Owen and Katz, 2005).

Table 2.8: A 2005 review of physical and chemical properties of human semen

Property	**Per 100mL**	**In average volume (3.4mL)**
Calcium (mg)	27.6	0.938
Chloride (mg)	142	4.83
Citrate (mg)	528	18.0
Fructose (mg)	272	9.25
Glucose (mg)	102	3.47
Lactic acid (g)	62	2.11
Magnesium (mg)	11	0.374
Potassium (mg)	109	3.71
Protein (g)	5.04	0.171
Sodium (mg)	300	10.2
Urea (g)	45	1.53
Urea (g)	16.5	0.561
Buffering capacity (β)	25	-
Osmolarity (mOsm)	354	-
pH	7.7	-
Viscosity (cP)	3-7	-
Volume (mL)	3.4	-

2.5.2 Appearance and Consistency of Human Semen

Semen is typically translucent with white, grey or even yellowish tint. Blood in the semen can cause a pink or reddish colour, known as haematospermia, and may indicate a medical problem. After ejaculation, the latter part of the ejaculated semen coagulates immediately, forming globules, while the earlier part of the ejaculate typically does not (Baker, 1993). After a period typically ranging from 15 – 30 minutes, prostate-specific antigen present in the semen causes the decoagulation of the seminal coagulum (Balk *et al*., 2003). It is postulated that the initial clotting helps keep the semen in the vagina, while liquefaction frees the sperm to make their journey to the ova (Gallup and Burch, 2004).

2.5.3 Semen Quality

Semen quality is a measure of the ability of semen to accomplish fertilization. Thus, it is a measure of fertility in a man. It is the sperm in the semen that is the fertile component, and therefore semen quality involves both sperm quantity and sperm quality (Owen and Katz, 2005).

2.5.4 Semen Quantity

The volume of semen ejaculate varies. A review of 30 studies concluded that the average was around 3.4 milliliters (ml), with some reporting amounts as high as 4.99 ml or as low as 2.3 ml (Owen and Katz, 2005). In a study with Swedish and Danish men, a prolonged interval between ejaculations caused an increase of the sperm count in the semen but not an increase of its amount (Richthoff *et al*., 2002).

2.5.5 Semen Storage

Semen can be stored in diluents such as the *Illini Variable Temperature* (IVT) diluent, which have been reported to be able to preserve high fertility of semen for over seven days. The IVT diluent is composed of several salts, sugars and antibacterial agents and gassed with CO_2. Semen cryopreservation can be used for far longer storage durations. For human sperm, the longest reported successful storage with this method is 21 years (Watson, 1993).

2.5.6 Heath Effect of Semen

In addition to its central role in reproduction, some studies have made claims that semen may have certain beneficial effects on human health:

a) Antidepressant: One study suggested that vaginal absorption of semen could act as an antidepressant; the study compared two groups of women, one of which used condoms and the other did not (Gallup *et al*., 2002; Jesse, 2010).
b) Cancer prevention: Studies suggested that seminal plasma might reduce breast cancer by "not less than 50 percent." This effect is attributed to its glycoprotein and selenium content, with apoptosis being induced by TGF-Beta. A related urban legend parodied these findings and claimed that performing fellatio at least three times a week reduced the risk of breast cancer (Monique *et al*., 1989).
c) Preeclampsia prevention: It has been hypothesized that substances in semen condition a mother's immune system to accept the "foreign" proteins found in sperm as well as the resulting fetus and placenta, keeping blood pressure low and thereby reducing the risk of preeclampsia (Koelman *et al*., 2000).

d) Increased libido: Another hypothesis has emerged that absorption of the testosterone contained in semen through a woman's vaginal walls during sexual intercourse (or even through the act of swallowing semen) may increase her sex drive (Jesse, 2010).

Other studies claim adversarial effects:

a) Cancer worsening: seminal plasma has prostaglandin elements that could accelerate the development of an already existing cervical cancer (Muller *et al*., 2006).

2.5.7 Semen and Transmission of Disease

Semen can be the vehicle for many sexually transmitted diseases, including HIV, the virus that causes AIDS. Further research, such as that by Mathur and Goust, demonstrated that non-preexisting antibodies were produced in humans in response to the sperm. These antibodies mistakenly recognized native T lymphocytes as *foreign* antigens, and consequently T lymphocytes would fall under attack by the body's B lymphocytes. Semen contains many proteins with potent antimicrobial activity against bacteria, fungi and virus. But none of these proteins are active against *Neisseria gonorrhoeae*, a common cause of sexually transmitted disease (Mathur *et al*., 1981).

2.5.8 Semen Allergy

In rare cases, people have been known to experience allergic reactions to seminal fluids, known as human seminal plasma hypersensitivity. Symptoms can be either localized or systemic, and may include vaginal itching, redness, swelling, or blisters within 30 minutes

of contact. They may also include generalized itching, hives, and even difficulty breathing (Guillet *et al*., 2005).

One way to test for human seminal plasma sensitivity is to use a condom during intercourse. If symptoms dissipate with the use of a condom, it is possible that sensitivity to semen is present. Mild cases of semen allergy can often be overcome by repeated exposure to seminal fluid (Weidinger *et al*., 2005). Scientists at Utrecht University studied the condition whereby some men "get flu-like symptoms such as feverishness, runny nose, extreme fatigue and burning eyes immediately after they ejaculate. Symptoms can last for up to week." This condition is termed post orgasmic illness syndrome (POIS), and it was discovered that this stemmed from an allergy to their own semen. Its effects could be cured using hyposensitization therapy or allergen immunotherapy (Weidinger *et al*., 2005).

2.5.9 Psychological Aspects of Seminal Fluid

A recent study has suggested that semen acts as an antidepressant in women, so that women physically exposed to semen are less likely to suffer from depression. It is thought that the psychological effects of semen are a result of its complex chemical make-up including several mood-altering hormones (testosterone, oestrogen, follicle-stimulating hormone, luteinizing hormone, prolactin and several different prostaglandins). In a scientific survey of 293 college women it was also found that those who did not use condoms were most likely to initiate sex and to seek out new partners as soon as a relationship ended, suggesting that the chemical dependency to semen creates a "rebound effect" (Gallup *et al*., 2002).

2.6 Spermatogenesis

Spermatogenesis is the process in which spermatozoa are produced from male primordial germ cells by way of mitosis and meiosis. The initial cells in this pathway are called spermatogonia, which yield primary spermatocytes by mitosis. The primary spermatocyte divides meiotically into two secondary spermatocytes; each secondary spermatocyte then completes meiosis as it divides into two spermatids. These develop into mature spermatozoa, also known as sperm cells. Thus, the primary spermatocyte gives rise to two cells, the secondary spermatocytes, and the two secondary spermatocytes by their subdivision produce four spermatozoa as shown in figure 2.4 (Song *et al*., 2011).

Spermatozoa are the mature male gametes in many sexually reproducing organisms. Thus, spermatogenesis is the male version of gametogenesis. In mammals it occurs in the male testes and epididymis in a stepwise fashion. Spermatogenesis is highly dependent upon optimal conditions for the process to occur correctly, and is essential for sexual reproduction. DNA methylation and histone modification have been implicated in the regulation of this process. It starts at puberty and usually continues uninterrupted until death; although a slight decrease can be discerned in the quantity of produced sperm with increase in age (Padubidri and Daftary, 2011; Song *et al*., 2011).

Figure 2.4 Various stages of spermatogenesis and the cells produced (Fishelson *et al*., 2007).

2.6.1 Importance of spermatogenesis

Spermatogenesis produces mature male gametes, commonly called sperm but specifically known as *spermatozoa*, which are able to fertilize the counterpart female gamete, the oocyte, during conception to produce a single-celled individual known as a zygote. This is the cornerstone of sexual reproduction and involves the two gametes both contributing half the normal set of chromosomes (haploid) to result in a chromosomally normal (diploid) zygote. To preserve the number of chromosomes in the offspring – which differs between species – each gamete must have half the usual number of chromosomes present in other body cells. Otherwise, the offspring will have twice the normal number of chromosomes, and serious abnormalities may result. In humans, chromosomal abnormalities arising from incorrect spermatogenesis can result in Down syndrome, Klinefelter's syndrome, and spontaneous abortion (Padubidri and Daftary, 2011).

2.6.2 Location of spermatogenesis

Spermatogenesis takes place within several structures of the male reproductive system. The initial stages occur within the testes and progress to the epididymis where the developing gametes mature and are stored until ejaculation. The seminiferous tubules of the testes are the starting point for the process, where stem cells adjacent to the inner tubule wall divide in a centripetal direction; beginning at the walls and proceeding into the innermost part, or lumen to produce immature sperm. Maturation occurs in the epididymis. For humans, entire process of spermatogenesis takes 74 days. Including the transport on ductal system, the process takes 3 months. Testes produce 200 to 300 million spermatozoa daily (Padubidri and Daftary, 2011).

2.6.3 Stages of spermatogenesis

The entire process of spermatogenesis can be broken up into several distinct stages, each corresponding to a particular type of cell in human. In the following Table 2.9, ploidy, copy number and chromosome/chromatid counts are for one cell, generally prior to DNA synthesis and division. The primary spermatocyte is arrested after DNA synthesis and prior to division (Fishelson *et al*., 2007).

Table 2.9 Stages of spermatogesnesis and cell division processes (Fishelson *et al*., 2007).

Cell type	**ploidy/chromosomes**	**DNA copy number/chromatids**	**Cell process**
Spermatogonium	diploid (2N) / 46	2C / 46	spermatocytogenesis (mitosis)
Primary Spermatocyte	diploid (2N) / 46	4C / 2x46	spermatidogenesis (meiosis 1)
Two Secondary Spermatocytes	haploid (N) / 23	2C / 46	spermatidogenesis (meiosis 1)
Four Spermatids	haploid (N) / 2	1C / 23	spermiogenesis
Four functional Spermatozoids	haploid (N) / 23	1C / 23	spermiation

2.6.3.1 Spermatocytogenesis

Spermatocytogenesis is the male form of gametocytogenesis and results in the formation of spermatocytes possessing half the normal complement of genetic material. In spermatocytogenesis, a diploid spermatogonium (figure 2.5) which resides in the basal

compartment of seminiferous tubules, divides mitotically to produce two diploid intermediate cells called primary spermatocytes. Each primary spermatocyte then moves into the ad-luminal compartment of the seminiferous tubules and duplicates its DNA and subsequently undergoes *meiosis I* to produce two haploid secondary spermatocytes, which will later divide once more into haploid spermatids. This division implicates sources of genetic variation, such as random inclusion of either parental chromosomes, or chromosomal crossover, to increase the genetic variability of the gamete (Fishelson *et al*., 2007).

Figure 2.5 Spermatocytogenesis of Spermatogonia to Secondary Spermatocyte (Fishelson *et al*., 2007).

Each cell division from a spermatogonium to a spermatid is incomplete; the cells remain connected to one another by bridges of cytoplasm to allow synchronous development. Not all spermatogonia divide to produce spermatocytes, so that the supply would not run out. Instead, certain types of spermatogonia divide to produce copies of themselves, thereby ensuring a constant supply of gametogonia to fuel spermatogenesis (Fishelson *et al*., 2007).

2.6.3.2 Spermatidogenesis

Spermatidogenesis is the creation of spermatids from secondary spermatocytes. Secondary spermatocytes produced earlier rapidly enter meiosis II and divide to produce haploid

spermatids. The brevity of this stage means that secondary spermatocytes are rarely seen in histological studies (Fishelson *et al*., 2007).

2.6.3.3 Spermiogenesis

During spermiogenesis, the spermatids begin to grow a tail, and develop a thickened mid-piece, where the microtubules gather and form an axoneme. Spermatid DNA also undergoes packaging, becoming highly condensed. The DNA is packaged firstly with specific nuclear basic proteins, which are subsequently replaced with protamines during spermatid elongation. The resultant tightly packed chromatin is transcriptionally inactive. The Golgi apparatus surrounds the now condensed nucleus, becoming the acrosome. One of the centrioles of the cell elongates to become the tail of the sperm. Maturation then takes place under the influence of testosterone, which removes the remaining unnecessary cytoplasm and organelles. The excess cytoplasm, known as *residual bodies*, is phagocytosed by surrounding Sertoli cells in the testes. The resulting spermatozoa are now mature but lack motility, rendering them sterile. The mature spermatozoa are released from the protective Sertoli cells into the lumen of the seminiferous tubule in a process called *spermiation* (Fishelson *et al*., 2007). The non-motile spermatozoa are transported to the epididymis in testicular fluid secreted by the Sertoli cells with the aid of peristaltic contraction. While in the epididymis the spermatozoa gain motility and become capable of fertilization. However, transport of the mature spermatozoa through the remainder of the male reproductive system is achieved via muscle contraction rather than the spermatozoon's recently acquired motility (Fishelson *et al*., 2007).

2.6.4 Role of Sertoli cells in spermatogenesis

At all stages of differentiation, the spermatogenic cells are in close contact with Sertoli cells which are thought to provide structural and metabolic support to the developing sperm cells. A single Sertoli cell extends from the basement membrane to the lumen of the seminiferous tubule (figure 2.6), although the cytoplasmic processes are difficult to distinguish at the light microscopic level (Xiao *et al*., 2013).

Figure 2.6 The organization of Sertoli cells (red) and spermatocytes (blue) in the testis. Spermatids which have not yet undergone spermination are attached to the lumenal apex of the cell (Xiao *et al*., 2013).

Sertoli cells serve a number of functions during spermatogenesis, they support the developing gametes in the following ways: (Xiao *et al*., 2013)

a) Maintain the environment necessary for development and maturation, via the blood-testis barrier
b) Secrete substances initiating meiosis
c) Secrete supporting testicular fluid
d) Secrete androgen-binding protein (ABP), which concentrates testosterone in close proximity to the developing gametes. Testosterone is needed in very high quantities

for maintenance of the reproductive tract, and ABP allows a much higher level of fertility

e) Secrete hormones affecting pituitary gland control of spermatogenesis, particularly the polypeptide hormone, inhibin
f) Phagocytose residual cytoplasm left over from spermiogenesis
g) They release Antimullerian hormone which prevents formation of the Müllerian Duct/Oviduct.
h) Protect spermatids from the immune system of the male, via the blood-testis barrier. The intercellular adhesion molecules ICAM-1 and soluble ICAM-1 have antagonistic effects on the tight junctions forming the blood-testis barrier. ICAM-2 molecules regulate spermatid adhesion on the apical side of the barrier.

2.6.5 Factors influencing spermatogenesis

The process of spermatogenesis is highly sensitive to fluctuations in the environment, particularly hormones and temperature. Testosterone is required in large local concentrations to maintain the process, which is achieved through binding of testosterone by androgen binding protein present in the seminiferous tubules. Testosterone is produced by interstitial cells, also known as Leydig cells, which reside adjacent to the seminiferous tubules. Seminiferous epithelium is sensitive to elevated temperature in humans and some other species, and will be adversely affected by temperatures as high as normal body temperature. Consequently, the testes are located outside the body in a sack of skin called the scrotum. The optimal temperature is maintained at 2 °C in man; 8 °C in mouse below body temperature. This is achieved by regulation of blood flow and positioning towards and

away from the heat of the body by the cremasteric muscle and the dartos smooth muscle in the scrotum. Dietary deficiencies (such as vitamins B, E and A), anabolic steroids, metals (cadmium and lead), x-ray exposure, dioxin, alcohol, and infectious diseases will also adversely affect the rate of spermatogenesis. In addition, the male germ line is susceptible to DNA damage caused by oxidative stress, and this damage likely has a significant impact on fertilization and pregnancy (Lewis and Aitken, 2005). Exposure to pesticides also affects spermatogenesis (Mehrpour and Karrari, 2014).

2.6.6 Hormonal control of spermatogenesis

Hormonal control of spermatogenesis varies among species. In humans the mechanism is not completely understood, however it is known that initiation of spermatogenesis occurs at puberty due to the interaction of the hypothalamus, pituitary gland and Leydig cells. If the pituitary gland is removed, spermatogenesis can still be initiated by follicle stimulating hormone and testosterone. Follicle-stimulating hormone stimulates both the production of androgen binding protein by Sertoli cells, and the formation of the blood-testis barrier. Androgen binding protein is essential to concentrating testosterone in levels high enough to initiate and maintain spermatogenesis, which can be 20–50 times higher than the concentration found in blood. Follicle stimulating hormone may initiate the sequestering of testosterone in the testes, but once developed only testosterone is required to maintain spermatogenesis. However, increasing the levels of follicle stimulating hormone will increase the production of spermatozoa by preventing the apoptosis of *type A spermatogonia*. The hormone inhibin acts to decrease the levels of follicle stimulating hormone. Studies from rodent models suggest that gonadotropin hormones (both LH and

FSH) support the process of spermatogenesis by suppressing the proapoptotic signals and therefore promote spermatogenic cell survival. The Sertoli cells themselves mediate parts of spermatogenesis through hormone production. They are capable of producing the hormones estradiol and inhibin. The Leydig cells are also capable of producing estradiol in addition to their main product testosterone (Pareek *et al*., 2007) .

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

Carbamazepine and Vitamin E of analytical grades were purchased from a Pharmaceutical store in Kaduna. 1% Eosin and 5% Negrosin stains were purchased from Cardinal Scientific Supply, Kaduna State, Nigeria. Physiological saline, distilled water, chloroform and 10% and 0.5% Formo-aldehyde, Xylene, 10% Phosphate buffer solution (PBS), 10% trichloroacetate (TCA) and 0.69% thiobabuturic acid (TBA) were obtained from Department of Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

3.1.2 Equipment

The following equipment and materials were used for the study:

Syringes and needles, metallic cages, oral canula measuring tape, masking tape, filter paper, disposable petri dishes, electronic balance, haemacytometer (Marienfield, Germany) and HM-Lux microscope (Germany), electronic analytical and precision balance (BA 210S, d=0.0001- Sartoriusen GA, Goettingen, Germany), erythrocyte diluting pipette, cover slips, glass slides, Pasteur dropper, Microplate reader PR5100 (Biorad, Austria), plane bottles, EDTA plane bottles, heparinized EDTA bottles, cotton wool, test tubes, tissue forceps, scalpel, surgical blades, scissors, digital vernial caliper (www.tresnainstrument.com),

centrifuging machine (Model: HNSII 2356157 1/7, made in United Kingdom) Spectrophotometers (Spectro V-16 (MRC) and Spectronic 20, and hotplate magnetic stirrer (Ultratect 532, made in United Kingdom).

3.1.3 Plant collection and identification

Calyces of *hibiscus sabdariffa* were purchased from Sabon Gari Market, Zaria, Nigeria. It was taxonomically identified and authenticated in the herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria by Mal. Umar Gaya, with a voucher number (1056) was deposited.

3.1.4 Experimental animal

Thirty adult male Wistar rats between the ages of 8-12 weeks, weighing from 120-180 g were used for this research. The animals were obtained from the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. The rats were housed in the animal house of the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University Zaria, Nigeria. The animals were kept in cages under normal environmental temperature (20 – 22 ^{o}C) and fed with standard pellet diet and water given *ad libitum*. The rats were allowed to acclimatize to the laboratory environment for one week before the experiment commenced and were grouped into five groups of six rats each and the treatment were administered through the oral route of administration using oral cannula.

3.2 METHODS

3.2.1 Preparation of the extraction

The *Hibiscus sabdariffs l*. calyces were washed thoroughly, dried in a shade and grounded into powder using pestle and mortar. The extraction of the *Hisbiscus sabdariffa l*. calyx was done using the method of cold maceration. Five hundred gram (500 g) of the powder calyx was dissolved in conical flask with water in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Science, Ahmadu Bello University, Zaria. The preparation was allowed to stand for 24 hours and filtered using filtration funnel. The filtrate was poured into an evaporating dish to evaporate the solvent in the extract over the water bath at the temperature of 70-75^{o}C (Trease and Evans, 1983), and a yield quantity of the crude extract was obtained.

3.2.2 Experimental Design

The rats were divided into two five groups, each comprising six animals.

Group I (n = 6 rats).

This group served as control. Rats were treated with normal saline orally for eight weeks.

Group II (n = 6 rats).

Rats in this group were treated with carbamazepine alone at a dose of 20 mg/kg daily (Marli *et al*., 2008) orally for eight weeks.

Group III (n = 6 rats).

This group was treated with carbamazepine (20 mg/kg) and *H. sabdariffa L.* (at a dose of 200 mg/kg) daily orally for eight weeks. *H. sabdariffa* was administered 30 minutes after carbamazepine treatment.

Group IV (n = 6 rats).

This group was treated with carbamazepine (20 mg/kg) and vitamin E (50 IU/kg body weight) (Santosh, *et al*., 2012) orally for eight weeks. Vitamin E was administered 30 minutes after carbamazepine treatment.

Group V (n = 6 rats).

This group was treated with carbamazepine, *H. sabdariffa L.* (at a dose of 200 mg/kg) and vitamin E (50 IU/kg body weight) orally for eight weeks. *H. sabdarriffa* and vitamin E (50 IU/kg body weight) were administered 30 minutes after carbamazepine treatment.

3.2.3 *Hibiscus sabdariffa* Dose Preparation and Administration

The stock concentration was prepared by dissolving 1 g of the extract in 2 ml of distilled water. The preparation was administered orally 200 mg/kg of body weight of rat for 8 weeks

3.2.4 *Hibiscus sabdariffa* Phytochemical Screening

Phytochemical screening of aqueous extract of *H. sabdariffa* calyx was conducted in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The method of Trease and Evans (1983) for phytochemical screening was adopted.

3.2.4.1 Test for carbohydrates

Molisch's test

A little quantity of the extract was placed in a clean test tube then few drops of molisch's reagent were added. Thereafter, a small quantity of concentrated sulphuric acid was allowed to run down the side of the test tube. A purple colour at the interphase was formed indicating the presence of carbohydrates (Trease and Evans, 1983; Harborne, 1998).

3.2.4.2 Test for cardiac glycosides

Kella-Killani's test

A little quantity of the extract was placed in a clean test tube; the extract was dissolved in glacial acetic acid. Few drops of ferric chloride were added. Then, 1 ml of concentrated sulphuric acid was run down the side of the test tube. A purple ring colour was seen at the interface, indicating the presence of cardiac glycosides (Harborne, 1998; Trease and Evans, 1983).

3.2.4.3 Test for anthraquinone derivatives

Borntrager's test

A little portion of the extract was put in a clean test tube soaked in 10 mls of benzene and filtered (in this case it was decanted since the solution of extract was aqueous). Then 5 ml of 10% ammonia solution was added to the filtrate and stirred. There was no pink-red or violet colour formation, indicating the absence of anthraquinone derivatives in the extract (Trease and Evans, 1983; Harborne, 1998).

3.2.4.4 Test for saponins

Frothing test

A small quantity of the extract was placed in a clean test tube and dissolved in 10ml of distilled water. The test tube was shaken vigorously for 30 minutes and allowed to stand for 30 minutes. A honey comb was formed for more than 30 minutes indicating the presence of saponins in the extract (Trease and Evans, 1983; Harborne, 1998).

3.2.4.5 Test for steroids and triterpenes

Liberman-Burchard's test

A little quantity of the extract was put in a test tube and an equal volume of acetic anhydrite was added to the extract. Then 1 ml of concentrated sulphuric acid was allowed to flow down the side of the test tube. A purple colour was observed immediately indicating the present of triterpenes (Trease and Evans, 1983; Harborne, 1998).

3.2.4.6 Test for flavonoids

Shinoda's Test

A little quantity of extract was placed in a test tube and mixed with 50% methanol. It was heated in a water bath. Metallic magnesium was added. Then four drops of concentrated hydrochloric acid was added. A red colour was formed indicating the presence of flavonoids (Trease and Evans, 1983; Harborne, 1998).

3.2.4.7 Test for tannins

Lead sub-acetate test

A little quantity of the extract was placed in a test tube and then 3 drops of lead acetate solution was added. A coloured precipitate was formed indicating the presence of tannins (Trease and Evans, 1983; Harborne, 1998).

3.2.4.8 Test for alkaloids

a) Mayer's Test

A few drops of Mayer's reagent were added to the extract in a test tube. No precipitate was formed, indicating the presence of alkaloids (Trease and Evans, 1983; Harborne, 1998).

b) Dragendroff's test

Few drops of Dragendroff's reagent were added to the extract in a test tube. No precipitate was formed, indicating the absence of alkaloids (Trease and Evans, 1983; Harborne, 1998;).

c) Wagner's test

A few drops Wagner's reagent were added to a small quantity of the extract in a clean test tube. No precipitate was formed, indicating the absence of alkaloids (Trease and Evans, 1983; Harborne, 1998).

3.2.5 Sample Collection

At the end of experiment four animals from each group were sacrificed by fenestration, 24 h after last day of administration. Blood samples were collected using test tubes and centrifuged at 2000 revolutions per minute. From the centrifuge, plasma was collected using needles and syringes and transferred into plain bottles. Orchidectomy was performed by open castration method. A midline or pre-scrotal incision was made and the testicles were milked out of the incision site. The testicles were exposed by incising the *tunica vaginalis* and removed. The testicular weights of each animal were evaluated with an electronic

analytical and precision balance (BA 210S, d=0.0001- Sartoriusen GA, Goettingen, Germany). One of the testes of each animal was fixed in 10% formol-saline for histological examination. Serum and the remaining testes of each animal were stored at -25°C for subsequent biochemical assays. The semen was obtained from the caudal epididymis. The method of collection as similar to that described by Akusu *et al*. (1985) and Oyeyemi and Ubiogoro (2005).

3.2.6 Sperm Motility

After sacrificing the animals, the cauda epididymis was removed and placed in a Petri-dish, containing 1 ml of PSS at room temperature. The epididymis was homogenized to allow the sperms to swim out. Then a drop of the mixture was placed on a glass slide using 2 ml syringe, the preparation was placed on a microscope (Saleem *et al*., 2013). Sperm motility was assessed as described by Freund and Carol (1993). The motility of epididymal sperm was evaluated microscopically within 2–4 min after the semen was obtained from the cauda epididymis, and data were expressed as percentages of progressively motile, none progressively motile and non-motile spermatozoa. The percentage of motility was evaluated visually at X 40 magnification (Sonmez *et al.*, 2007).

3.2.7 Sperm Count

After sacrificing the animals, the cauda epididymis was removed and placed in a Petri-dish, containing 1 ml of PSS at room temperature (22 ^{o}C). The epididymis was homogenized to allow the sperms to swim out. One millilitre of 0.5% formolsaline was added to the homogenate and mixed in the petridish. The preparation was drawn with red blood cell pipette and allowed to settle down for about 10 minutes before introducing into

haemocytometer. The spermatozoa were counted by haemocytometer using the improved Neubauer (0.0025mm^2, Marienfeld, Germany) chamber. The total epididymal sperm per ml was calculated as follows. Total epididymal sperm/ml = (Average number of sperm per chamber) x 10^3 x (Dilution Factor) (Saleem *et al*, 2013).

3.2.8 Sperm Morphology

The epididymis was minced in 1 ml PSS to make a suspension. Sperm morphology was determined by the method of Melissa and Rouge, (2006). Briefly, microscope slides and nigrosin-eosin stains were pre-warmed to body temperature. A drop of stain was made onto the end of a slide and a small droplet of semen was pipette next to the stain. The edge of another slide was placed inside the drops of stain and semen, and the slide was rocked back and forth a few times to mix the sperm and stain. A second side was smeared across the surface of the first. The second slide was dried by waving it back and forth in the air. The slide was examined using a bright field microscope using X 40 objective lens. Sperm head morphology was classified into normal and abnormal types. The abnormal spermatozoa were classified into two categories: (1) spermatozoa with defective heads (amorphous, hook less, banana shaped, double head, microcephaly and cephalo-caudal junction defects; and (2) Spermatozoa with defective tails (two-tailed, coiled/bent tails) (Vijay *et al.*, 2013).

3.2.9 Preparation of Testicular Homogenate

A testis of each animal labeled according to the groups was removed from storage (freezer) and allowed to defreeze. The testes was homogenized in 10 ml phosphate buffer solution (pH 7.4) using a pestle and mortar. The homogenate formed was poured into test tubes labeled according to the group and number of each animal. The resultant homogenates were

centrifuged at 2,000 revolutions per minute for 10 minutes at 4°C. The supernatant was collected and used for the experiments within 24 hours.

3.3 ANTIOXIDANT ENZYME ANALYSIS

3.3.1 Determination of Testicular Enzymatic Antioxidants

3.3.1.1 Assay of catalase (CAT) activity

The CAT activity was assessed using NWLSS CAT activity assay kit (Product NWK-CAT01, Specificity: Catalase (sensitivity: 6.0 U Catalase/mL). Catalase enzyme activity was measured based on the principle of catalase consumption of H_2O_2 substrate at 240 nm (Beers and Sizer, 1952).

3.3.1.2 Assay of superoxide dismutase activity

Activity of SOD in the rat serum was determined using NWLSS SOD assay kit (Product NWK-SOD02, Specificity: Cu/Zn, Mn and Fe Superoxide Dismutase, Sensitivity: 5 U/mL). The assay kit is based on the principle of superoxide inhibition of auto-oxidation of haematoxylin as described by Martin *et al*. (1987).

3.3.1.3 Assay of glutathione peroxidase activity

The activity of glutathione peroxidase was assessed using NWLSSTM cGPx (GPx1) ELISA assay kit (Product NWKGPX02, Specificity: Glutathione peroxidase, Sensitivity: 12.5 pg/ml). The NWLSS™ cGPx Assay was based on a sandwich enzyme-linked immunosorbent assay (ELISA), where sample GPx concentration was determined by comparing the 450 nm absorbance of sample wells to the absorbance of known standards (Takebe, 2002).

3.4 ASSESSMENT OF LIPID PEROXIDATION

Malondialdehyde (MDA) level was estimated in the testicular homogenate according to the method of Burge and Aust (1987). Thiobarbituric acid (TBA) reacted with malondialdehyde (MDA) in acidic medium at temperature 95^0C for 10 min to form thiobarbituric acid reactive substance (TBARs). In brief, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 ml of TBA-TCA-HCl (1:1:1 ratio) reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA), and placed in water bath for 15 min and then cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was calculated using the molar absorptivity of MDA which was 1.56 x105 $M^{-}1$ $cm^{-}1$ and expressed as nmol/mg protein.

3.5 ASSESSMENT OF SERUM LEVELS OF TESTOSTERONE

Testosterone serum level was determined by enzyme immunoassay competitive binding principle technique using testosterone kit (Syntron Bioresearch, Inc. Carlsbad, U.S.A Microwell Testosterone EIA). Testosterone present in the sample competed with enzyme-labeled testosterone for binding with anti-testosterone antibody immobilized on the microwell surface. The amount of conjugate that bound to the microwell surface was decreased in proportion to the concentration of testosterone in the sample. The testosterone concentration in the sample and the control was determined from a standard curve, using the method of Wilkle and Utley, (1987).

3.7 Histological procedures

After the extraction of the testis from the rats, the organ was promptly and adequately treated with 10% formaldehyde (fixation) in order to preserve its structure and molecular composition. After fixation, the piece of organ was dehydrated by bathing it successfully in graded mixture of ethanol and water (70 – 100%). The ethanol was then replaced with a solvent miscible with the embedding medium. As the tissues were infiltrated with xylene, it became transparent (clearing). The impregnated tissue by xylene was placed in melted paraffin in an oven, maintained at 58 – 60^{o}C (embedding). The heat caused the solvent to evaporate and the spaces within the tissues became filled with paraffin. The tissue together with its impregnating paraffin hardened after removal from the oven. The sections (5 μm) were then floated on water and transferred to a glass slide, and stained with haematoxylin and eosin stains. The slides were viewed under light microscope with magnification x 100.

3.8 DATA ANALYSIS

Data obtained from the study were expressed as mean ± SEM. The differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test. Graphpad Prism statistical package, San Diego, Califonia, U.S.A (www.Graphpad.Com) was used for the analyses. Values of $P < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical analysis of the aqueous extract of *Hibiscus sabdariffa*

Phytochemical analysis of the aqueous extract of *Hibiscus sabdariffa* revealed the presence of the following components: alkaloids, tannins, saponins, carbohydrates, cardiac glycosides, steroids and triterpenes, and flavonoids. However, a negative reaction was obtained for anthraquinones (Table 4.1).

Table 4.1: Phytochemical Constituents of the Aqueous Extract of *Hibiscus sabdariffa*.

Constituents	**Inference**
Alkaloids	+
Tannins	+
Carbohydrates	+
Cardiac glycosides	+
Steroids and triterpenes	+
Anthraquinones	–
Flavonoids	+
Saponins	+

+ = positive (present)

- = negative (absent)

4.2 Effect of treatments on epididymal sperm count

Figure 4.1 shows the effect of treatments on epididymal sperm count. Sperm count was significantly ($P < 0.05$) higher in CBZ + HS + VE group ($89.00 \pm 9.85 \times 10^6$/ml) than in any other group (control: $61.50 \pm 1.44 \times 10^6$/ml; CBZ: $49.75 \pm 3.19 \times 10^6$/ml; CBZ + HS: $71.25 \pm 7.08 \times 10^6$/ml; CBZ + VE: $63.00 \pm 2.67 \times 10^6$/ml).

Figure 4.1 Effects of treatments on epididymal sperm count in adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.3 Effect of treatments on percentage progressive sperm motility

Figure 4.2 shows the effect of treatments on progressive sperm motility in the groups. Percentage progressive sperm motility was significantly higher ($P < 0.05$) in CBZ + HS, CBZ + VE and CBZ + CBZ + HS +VE groups (66.25 ± 6.88%, 67.50 ± 6.29% and 82.25 ± 4.78%, respectively) than in control and CBZ groups (61.25 ± 3.14% and 42.50 ± 4.78%, respectively).

Figure 4.2 Effect of treatments on progressive sperm motility in adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.4 Effects of treatment on percentage non-progressive sperm motility

Figure 4.3 shows the effect of treatments on non-progress sperm motility in the groups. Percentage non-progressive sperm motility was significantly ($P < 0.05$) lower in CBZ + HS +VE group than in the control, CBZ, CBZ + HS and CBZ + VE groups (7.50 ± 1.44% versus 26.25 ± 2.39%, 30.00 ± 4.56%, 20.00 ± 3.53% and 17.50 ± 2.50%, respectively).

Figure 4.3 Effect of treatments on non-progressive sperm motility in adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.5 Effect of treatments on percentage non-motile sperm

Figure 4.4 shows the effect of treatments on percentage non-motile sperm in the groups. Percentage non-motile sperm was significantly lower ($P < 0.05$) in CBZ + HS + VE group than in control, CBZ, CBZ + HS and CBZ + VE groups (1.00 ± 3.53% versus 22.50 ± 4.78%, 27.50 ± 1.44%, 13.75 ± 3.75% and 15.00 ± 5.00%, respectively).

Figure 4.4 Effect of treatments on non-motile sperms in adult male Wistar rats

[a, b] = Means with different superscript letters are significantly ($P < 0.05$) different

4.6 Effect of treatments on testosterone concentration

Figure 4.5 shows the effect of treatments on serum testosterone concentration. Serum testosterone concentration was higher ($P < 0.05$) in CBZ + HS + VE group (5.97 ± 0.31%) than in any other group (control: 6.15 ± 0.22%; CBZ: 5.05 ± 0.06%; CBZ + HS: 4.25 ± 0.34% and CBZ + VE: 4.25 ± 0.34%, respectively).

Figure 4.5 Effects of treatments on serum testosterone concentration in adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.7. Testicular Malondialdehyde Concentration

Figure 4.6 represents changes in testicular MDA concentrations in control group and treatment groups. Testicular MDA concentration was significantly ($P < 0.05$) lower in CBZ + HS, CBZ +VE and CBZ + HS + VE groups than in the control and CBZ groups (0.04 ± 0.02 nmoL/mg of tissue protein; 0.06 ± 0.00 nmoL/mg tissue protein; 0.02 ± 0.00 nmoL/mg tissue protein VS 0.05 ± 0.01 nmoL/mg tissue protein and 0.17 ± 0.01 nmoL/mg tissue protein, respectively).

Figure 4.6 Changes in testicular Malondialdehyde levels in control and treatment groups of adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.8.1 Superoxide dismutase

Figure 4.7 shows changes in SOD activity in control and treatment groups. Testicular SOD levels were significantly ($P < 0.05$) higher in CBZ + VE and CBZ + HS + VE groups than in control, CBZ and CBZ + HS groups (2.42 ± 0.08 IU/L, 2.22 ± 0.02 IU/L VS 2.07 ± 0.07 IU/L, 1.87 ± 0.04 IU/L and 1.82 ± 0.07 IU/L, respectively)

Figure 4.7: Testicular superoxide dismutase activity in control and treatments groups of adult male Wistar rats

$^{a, b, c, d}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.8.2 Glutathione peroxidase activity

Figure 4.8 shows GPx activities in control group and treatment groups. There was no significant difference in testicular glutathione peroxidase levels between the control and treatment groups (Control: 62.2 ± 1.79 IU/L versus CBZ: 54.7 ± 2.56 IU/L, CBZ + HS: 53.0 ± 1.22 IU/L, CBZ + VE: 58.5 ± 0.86 IU/L and CBZ + HS + VE: 61.7 ± 1.43 IU/L, respectively; $P > 0.05$).

Figure 4.8 Changes in testicular glutathione peroxidase activity in control and treatment groups of adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.8.3 Catalase activity

Figure 4.9 shows testicular CAT levels in control and treated adult male Wistar rats. There was no significant ($P > 0.05$) difference in testicular CAT levels between the control and treatment groups (Control: 62.75 ± 2.49 IU/L, CBZ: 62.0 ± 0.91 IU/L, CBZ + HS: 58.7 ± 1.25 IU/L, CBZ +VE: 57.7 ± 1.49 IU/L and CBZ + HS + VE: 61.75 ± 1.25 IU/L, respectively).

Figure 4.9 Changes in testicular catalase activity in control and treated groups of adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.9 Effects of treatments on testicular histology

4.9.1 Control group

The effect of treatments on testicular histology reveals normal histo-architecture of testis in the group treated with normal saline (Plate II).

4.9.2 CBZ group

The effect of treatments on testicular histology in the group treated with CBZ revealed disorganization of the normal seminiferous tubules, degeneration of the epithelia cell, presence of vacuolation between the epithelia cells and the germ cell layers, and few mature central spermatozoa (Plate III).

4.9.3 CBZ + HS group

The effect of treatments on testicular histology in the group treated with CBZ + HS revealed pronounced restoration of damaged germ cell layers and slight reduction of spermatozoa in the lumen of the seminiferous tubule (Plate IV).

4.9.4 CBZ + VE group

The effect of treatments on testicular histology in the group treated with CBZ + VE revealed mild degeneration of the germ cell layers and the presence of a few spermatozoa in the lumen of the seminiferous tubule (Plate V).

4.9.5 CBZ + HS + VE group

The effect of treatments on testicular histology in the group treated with CBZ + HS + VE showed less damage of the germ cells and the epithelia cells were preserved (Plate VI).

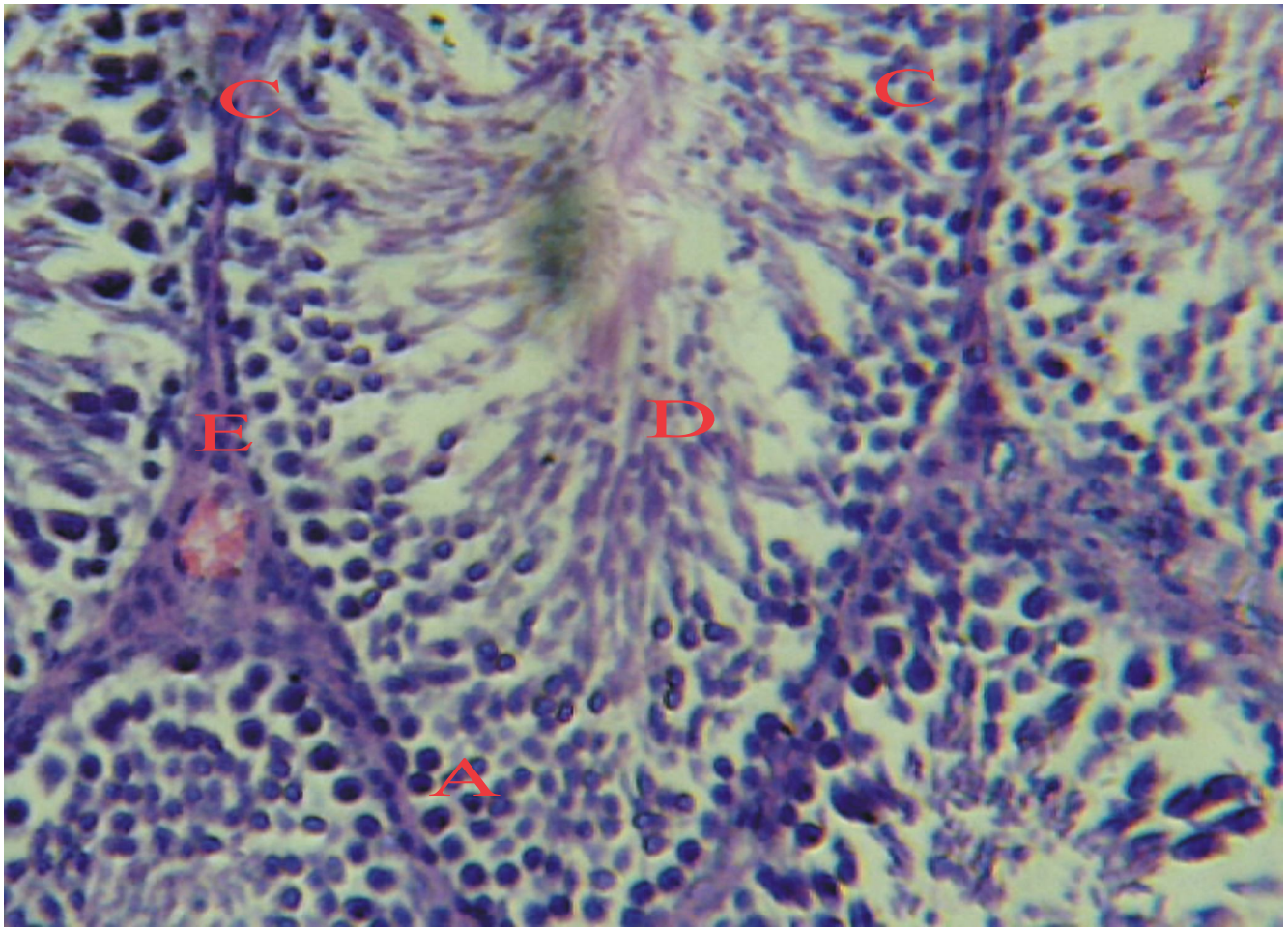


Plate II: Photomicrograph of the testis of an adult male Wistar rat treated with normal saline. Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 100.

Plate III: Photomicrograph of the testis of an adult male Wistar rat treated with carbamazepine. Note the degeneration of germinal epithelia cells (A), slight vacuolation appearing between the epithelium and the spermatogenic cells and few matured spermatozoa and degenerative germ cells (B) in the central lumen (C). H & E X 100

Plate IV: Photomicrograph of the testis of an adult male Wistar rat treated with carbamazepine + *Hibiscus sabdariffa*. Note the scanty germ cell layer (A), spermatocytes layer (B) and spermatozoa layer (C) close to the tubular lumen (D). H & E X 100

Plate V: Photomicrograph of the testis of an adult male Wistar rat treated with carbamazepine + vitamin E. Note the reduction in the number of germ cell layer (A), spermatocyte cells layer (B) and central spermatozoa layer (D) in the seminiferous tubular lumen (E) and enlargement of interstitial cell of Leydig (C) and interstitial spaces (F). H & E Mag. X 100

Plate VI: Photomicrograph of the testis of an adult male Wistar rat treated with carbamazepine + *Hibiscus sabdariffa* + vitamin E. Note the relatively normal spermatogonia layer (B) lining the basement membrane (A), spermatocyte layer (C), and spermatozoa layer (D) close to the center of the seminiferous tubular lumen (E). H & E X 100

4.10 Effects of treatment on sperm morphology

Figure 4.15 shows the effect of treatments on sperm morphology. There were no significant ($P > 0.05$) differences in the head and mid-piece abnormalities between the control and treatment groups. However, a significant difference was observed in normal sperm morphology and tail abnormalities between treatment groups. Percentage normal sperm morphology was higher in control; CBZ + HS, CBZ + VE and CBZ + HS + VE groups than in the CBZ group (92.75 ± 0.47%, 91.50 ± 0.64%, 90.0 ± 0.57%, 92.25 ± 0.35% VS 86.75 ± 0.62%, respectively $P > 0.05$). Similarly, sperm tail abnormalities were significantly lower in control, CBZ + HS, CBZ + VE and CBZ + HS + VE groups than in the CBZ group (7.25 ± 0.47%, 8.50 ± 0.64%, 10.0 ± 0.57%, 7.75 ± 0.35% VS 13.25 ± 0.62%, respectively; $P < 0.05$).

Figure 4.15: Effect of treatments on sperm morphology of adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.11 Effect of treatments on testicular weight

Figure 4.17 shows the effect of treatments on right and left testicular weights. There were no significant ($P > 0.05$) except CBZ + VE changes in the weight of right and left testes between control and treatment groups.

Figure 4.17 Effects of treatments on right and left testicular weight of adult male Wistar rats

4.12 Effects of treatment on right and left testicular length

Figure 4.19 shows the effect of treatments on right and testicular length. There were no significant ($P > 0.05$) changes in the lengths of right and left testes between the control and treatment groups.

Figure 4.18 Effect of treatments on testicular length of right and left testes in adult male Wistar rats

4.13 Effects of treatment on right and left testicular circumference

Figure 4.21 shows the effects of treatment on right and left testicular circumference. There were no significant ($P > 0.05$) changes in the circumference of the right and left testes between the control and treatment groups.

Figure 4.19 Effect of treatments on testicular circumference of the right and left testes of adult male Wistar rats

CHAPTER FIVE

5.0 Discussion, Summary, Conclusion and Recommendation

5.1 DISCUSSION

The median lethal dose of HS in the present studies was found to be greater than 5000mg/kg. This was within the range as reported by other researchers, indicating that the plants extract is not acutely toxic (Bako *et al*., 2009).

The phytochemical screening of the extract revealed the present of alkaloids, carbohydrates, cardiac glycosides, saponins, steroid and triterpenes, flavonoids, and tannins in the extract. These phytochemical compounds are known to play important roles in bioactivity of medicinal plants. Flavonoids which are part of the phytochemical constituents of *Hibiscus sabdariffa* exhibit a wide range of biological activities, one of which is their ability to scavenge for hydroxyl radicals, and superoxide anion radicals, and thus health-promoting action (Ferguson, 2001). Flavonoids also exhibit anti-inflammatory, antiangionic, anti-allergic, analgesic and antioxidant properties (Hodek *et al*., 2002).

The present study showed a significant reduction in epididymal sperm concentration in the CBZ group as compared to other groups, which agreed with the result obtained by Leskiewicz *et al*. (2008). The lower sperm count in the CBZ group could be partly due to the induction of oxidative stress in the testes and the spermatozoa as reported by earlier workers (Gallup *et al*., 2002; Isojarvi *et al*., 2004; Olive and Miraglia, 2009). The spermatozoa are highly susceptible to damage from ROS due to high concentration of polyunsaturated fatty acids within their plasma membrane (Krishnamoorthy *et al*., 2007), high production of free radicals, resulting from mitochondrial respiration and deficient DNA

repair mechanism (Zini *et al*., 1993). Although, spermatozoa require a short exposure to low amount of free radicals for sperm capacitation and to become hyperactive (Mariana *et al*., 2009), ROS must be continuously inactivated to a level that will not be injurious to the spermatozoa. Spermatozoa possess an array of antioxidant defence mechanisms such as SOD, CAT and GPx (Alverex and Storey, 1989). Anti-epileptic drugs like CBZ are capable of increasing oxidative challenge, which can be neutralized by an array of antioxidant mechanisms in the body. When the activities of antioxidant enzymes and molecules are overwhelm, oxidative stress ensures. The resulting excess free radicals damage lipid and protein components of cell membranes. This leads to acceleration of germ cell apoptosis, resulting in a decline in sperm count and alteration of gonadal integrity and function (Agarwal *et al*., 2003).

The Sertoli cell is a major determinant of sperm output in the testes (Sharpe *et al*., 2003). The reduced sperm count seen in this study in the CBZ group may be due to the reduced testosterone concentration possibly due to low LH and oxidative damage to the Leydig cells, consistent with the finding of Shetty *et al*. (2007). The net effect of low testosterone levels is decreased spermatogenesis and androgen-dependent epididymal sperm maturation (Krishnamoorthy *et al*., 2007). The present study has shown that treatment with the aqueous extract of HS and VE, ameliorated the CBZ-induced low sperm count. This may be due to anti-oxidant activities of the extract and VE apparently acting synergistically to reduce free radical load in the testes. The increase in the sperm concentration in the groups co-treated with the HS and VE may be due to decreased lipoperoxidative damage to the testicular tissues. Also, the amelioration of oxidative damage to the testes by HS and VE may have enhanced LH production, hence increasing the testosterone production by Leydig cells.

The present study showed a marked decrease in the sperm motility in the group exposed to CBZ compared to the control and other treated groups. This result agreed with previous findings by Shen and Chen (1990), who reported that carbamazepine has membrane stabilizing effect and inhibit human sperm motility. The reduced sperm motility in both progressive and non-progressive movement seen in this study may be ascribed to accumulation of proteins in the testes and epididymis, secondary to androgen deprivation, resulting in increased formation of abnormal spermatozoa (Rao and Chinoy, 1983). Sperm DNA damage occusr secondary to oxidative stress, exacerbated by a decline in local antioxidant protection, especially during epididymal maturation. This results in variety of adverse clinical outcomes (Aitken and De Iuliss, 2004). Hence, a cascade of defective spermiogenic processes with characteristic retention of excess residual cytoplasmic, persistent nuclear histones, and poor zona binding and disrupt chaperone content, causing formation of dysmature cells (Aitken and De Iuliss, 2004).

In the current study, there was a relatively higher testosterone level in the control as compared to the treated groups. The lower serum testosterone concentration in the CBZ group may be linked to the inhibitory effect of CBZ on the secretion of pituitary gonadotropins (FSH and LH), which aid in testosterone biosynthesis (Joshi *et al*., 2007). Similarly, decrease in testosterone concentration seen in the CBZ-treated group may occur due to direct damage to Leydig cells (Oliva and Miraglia, 2009). Therefore, the low testosterone concentration in the CBZ-treated group as compared to the control group may be due to deleterious effect of CBZ on both the brain and the testicular tissues due to oxidative damage. The improvement seen in the groups co-treated with HS and VE suggests an ameliorative effect of HS and VE on CBZ-induced testicular and sperm defects.

Serum MDA level is an index of free radical generation, which increases in conditions of oxidative stress (Kehrer, 1993). The result obtained in this present study indicates significantly higher MDA level in CBZ-treated as compared to the control rats. This is in conformity with previous findings by Tarun *et al*. (2010). Malondialdehyde levels in the groups treated with CBZ + HS, and CBZ + VE were significantly lower than in the CBZ treated group. This is suggestive of a compensatory mechanism by the testicular antioxidant enzymes, induced by the synergic effect of HS and VE to reduce the generation of free radicals; thus, ameliorating oxidative stress in the testes.

Antioxidant status of the testes in this study shows significant increase in antioxidant enzymes (SOD and GPx) activities in the groups treated with CBZ + VE and CBZ + HS + VE. These findings were consisted with the report of Arm and Alaa-Eldin (2006). The antioxidant enzymes play a crucial role in the cellular defence mechanism against ROS (Bernabucci *et al*., 2002). Superoxide dismutase serves as the first line of defense against the deleterious effect of ROS. The function of intracellular SOD is to scavenge superoxide (O_2-), produced by cellular metabolism, by catalyzing dismutation of superoxide to oxygen (O_2) and hydrogen peroxide (H_2O_2) (Bernabucci *et al*., 2002; Das, 2011). The increased dismutation of O_2- by SOD leads to increase in production of H_2O_2, which is further detoxified to H_2O and O_2 by CAT and GPx. The potent antioxidant activity of *Hibiscus sabdariffa* obtained in the present study is in agreement with the finding of Kabele *et al*. (2013), who reported the plant possess a potent intrinsic ability to scavenge free radicals (Kabele *et al*., 2013).

Histological examination of the testes of rats treated with CBZ revealed degeneration in spermatogonia cells, lining the seminiferous tubule, associated with incomplete

spermatogenesis when compared with the control group treated with normal saline. The degeneration of germ cells and abnormal interstitial spaces with degeneration of Leydig cells in CBZ treated group seen in this study may due to the cytotoxic effect of CBZ. This agrees with the findings of Shetty and Naryana (2007), who reported that CBZ depresses spermatogenesis, which results in the death of immature germ cells present in the seminiferous epithelium. Improvements in the histopathological pictures with preservation of testicular integrity that were observed from rats treated with CBZ + HS and CBZ + VE could be ascribed to the antioxidant effects of HS and VE. This is consistent with the findings of Dare *et al*. (2012), who showed that flavonoids, which are part of the phytochemical constituents of HS, exhibit a wide range of biological activities, especially their ability to scavenge for hydroxyl and superoxide anion radicals. The CBZ + HS + VE group had relatively normal seminiferous tubule and germ cells, when compared to the CBZ, CBZ + HS and CBZ + VE treated groups. This result suggests better ameliorative effect of HS + VE as compared with co-treated HS or VE alone.

The present study revealed no significant difference in sperm head and mid-piece abnormalities, but a significant difference in sperm tail abnormalities in CBZ + HS, CBZ + VE and CBZ + HS + VE treated groups was recorded, compared to the control and CBZ treated groups. In the group exposed to CBZ only, relatively higher sperm morphological abnormalities were observed as compared to other treated groups. This finding agrees with the earlier report by Shetty and Naryana (2007), that the abnormalities seen may be due to alterations in the process of sperm maturation, where sperm cells develop from damaged seminiferous tissue, which may have occurred as a result of the lipoperoxidative damage induced by CBZ exposure on the testicular tissues.

5.2 SUMMARY

The present study has demonstrated that sub-chronic CBZ administration to male Wistar rats for 8 weeks caused decrease in sperm concentration, sperm motility, serum testosterone level, and relative decrease in testicular SOD, GPx and CAT, and an increase in testicular MDA. The present study also demonstrated that administration of HS and vitamin E ameliorated the oxidative stress and histological alterations in the testes caused by CBZ treatment. These deleterious effects by CBZ that were ameliorated by HS and VE resulted from increased compensatory process, evidenced by high activities of antioxidant enzymes in groups treated with HS and vitamin E.

5.3 CONCLUSION

In conclusion, the results obtained from the present study demonstrated that CBZ treatment induced oxidative stress in the testes of Wistar rats. *Hibiscus sabdariffa* and vitamin E or their combination ameliorated carbamazepine-induced oxidative stress responses in the testes of Wistar rats.

5.4 RECOMMENDATION

Based on the finding of this study, the followings are recommended:

I. The use of HS as an alternative antioxidant alone or with VE in long –term use of CBZ.

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APPENDIX I

Sperm count and percentage progressive, non-progressive and non-motile sperm cells in control and treated adult male Wistar rats

SPERM PARAMETERS	CONTROL	CBZ (20 mg/kg)	CBZ + HS (200 mg/kg)	CBZ + VE (50 IU/kg)	CBZ + HS + VE
Sperm cell (x10^6/ml)	61.50 ± 1.44^a	49.75 ± 3.19^b	71.25 ± 7.08^c	63.00 ± 2.67^b	89.00 ± 9.85^c
Prog. Sperm cell (%)	61.25 ± 3.14^a	42.50 ± 4.78^b	66.25 ± 6.88^b	67.50 ± 6.29^b	82.50 ± 4.78^c
Non-prog. Sperm cell (%)	26.25 ± 2.39^a	30.00 ± 4.56^a	20.00 ± 3.53^b	17.50 ± 2.50^b	7.50 ± 1.44^c
Non motile sperm Cell (%)	22.50 ± 4.78^a	27.50 ± 1.44^a	13.75 ± 3.75^b	15.00 ± 5.00^b	10.00 ± 3.53^b

Data were expressed as Mean ± SEM. N = 4

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

APPENDIX II

Percentage normal sperm morphology and sperm abnormalities in control and treated adult male Wistar rats

SPERM MORPHOLOGY CONTROL CBZ (20 mg/kg) CBZ + HS (200 mg/kg) CBZ + VE (50 IU/kg) CBZ + HS + VE

Normal sperm cells (%)	92.75 ± 0.47^{a}	86.75 ± 0.62^{b}	91.50 ± 0.64^{a}	90.0 ± 0.57^{c}	92.25 ± 0.35^{a}
Abnormal sperm cells (%)	7.25 ± 0.47^{a}	13.25 ± 0.62^{b}	8.50 ± 0.64^{c}	10.0 ± 0.57^{d}	7.75 ± 0.35^{a}

Data were expressed as Mean ± SEM. N = 4

$^{a, b, c, d}$ = Means with different superscript letters are significantly ($P < 0.05$) different

APPENDIX III

Levels of serum testosterone and testicular biomarkers of oxidative stress in control and treated adult male Wistar rats

TESTICULAR BIOMARKER	CONTROL	CBZ (20 mg/kg)	CBZ + HS (200 mg/kg)	CBZ + VE (50 IU/kg)	CBZ + HS + VE
Testosterone (µg/ml)	6.15 ± 0.22^{a}	5.05 ± 0.06^{b}	4.25 ± 0.34^{c}	4.70 ± 0.16^{c}	5.97 ± 0.31^{a}
Superoxide Dismutase	2.07 ± 0.07^{a}	1.87 ± 0.04^{b}	1.82 ± 0.07^{b}	2.42 ± 0.08^{c}	2.22 ± 0.02^{d}

(IU/L)				
Glutathione Peroxidase	62.2 ± 1.79[a]	54.7 ± 2.56[b]	53.0 ± 1.22[b]	58.5 ± 0.86[c] 61.7 ± 1.43[a]
(IU/L)				
Catalase	62.75 ± 1.09[a]	62.0 ± 0.91[b]	58.7 ± 1.05[c]	57.7 ± 1.09[c] 61.5 ± 1.05a[b]
(IU/L)				
Malondialdehyde	0.05 ± 0.01[a]	0.17 ± 0.012[b]	0.04 ± 0.02[c]	0.06 ± 0.004[c] 0.02 ± 0.004[d]
(nmoL/mg)				

Data were expressed as Mean ± SEM. N = 4

[a, b, c, d] = Means with different superscript letters are significantly ($P < 0.05$) different